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(54) Title: ANALYSIS OF GENETIC POLYMORPHISMS AND GENE COPY NUMBER			
(57) Abstract			
<p>The invention provides methods for detecting variations in polymorphic sites and/or variations in gene copy number. The methods are particularly useful for analysis of biotransformation genes, such as cytochromes P450.</p>			

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ANALYSIS OF GENETIC POLYMORPHISMS AND GENE COPY NUMBERSTATEMENT OF GOVERNMENT INTEREST

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5 certain rights to the invention.

BACKGROUND OF THE INVENTIONFIELD OF THE INVENTION

The present invention resides in the field of molecular genetics and diagnostics.

10 DESCRIPTION OF RELATED ART

Virtually all substances introduced into the human body (xenobiotics) as well as most endogenous compounds (endobiotics) undergo some form of biotransformation in order to be eliminated from the body. Many enzymes contribute to
15 the phase I and phase II metabolic pathways responsible for this bioprocessing. Phase I enzymes include reductases, oxidases and hydrolases. Among the phase I enzymes are the cytochromes P450, a superfamily of hemoproteins involved in the oxidative metabolism of steroids, fatty acids,
20 prostaglandins, leukotrienes, biogenic amines, pheromones, plant metabolites and chemical carcinogens as well as a large number of important drugs (Heim & Meyer, *Genomics* 14, 49-58 (1992)). Phase II enzymes are primarily transferases responsible for transferring glucuronic acid, sulfate or
25 glutathione to compounds already processed by phase I enzymes (Gonzales & Idle, *Clin. Pharmacokinet.* 26, 59-70 (1994)). Phase II enzymes include epoxide hydrolase, catalase, glutathione peroxidase, superoxide dismutase and glutathione S-transferase.

30 Many drugs are metabolized by biotransformation enzymes. For some drugs, metabolism occurs after the drug has exerted its desired effect, and result in detoxification of the drug and elimination of the drug from the body. Similarly, the biotransformation enzymes also have roles in detoxifying
35 harmful environmental compounds. For other drugs, metabolism

is required to convert the drug to an active state before the drug can exert its desired effect.

Genetic polymorphisms of cytochromes P450 and other biotransformation enzymes result in phenotypically-distinct subpopulations that differ in their ability to perform biotransformations of particular drugs and other chemical compounds. These phenotypic distinctions have important implications for selection of drugs. For example, a drug that is safe when administered to most human may cause intolerable side-effects in an individual suffering from a defect in an enzyme required for detoxification of the drug. Alternatively, a drug that is effective in most humans may be ineffective in a particular subpopulation because of lack of a enzyme required for conversion of the drug to a metabolically active form. Further, individuals lacking a biotransformation enzyme are often susceptible to cancers from environmental chemicals due to inability to detoxify the chemicals. Eichelbaum et al., *Toxicology Letters* 64/65, 155-122 (1992). Accordingly, it is important to identify individuals who are deficient in a particular P450 enzyme, so that drugs known or suspected of being metabolized by the enzyme are not used, or used only with special precautions (e.g., reduced dosage, close monitoring) in such individuals. Identification of such individuals is also important so that such individuals can be subjected to regular monitoring for the onset of cancers.

Existing methods of identifying deficiencies are not entirely satisfactory. Patient metabolic profiles are currently assessed with a bioassay after a probe drug administration. For example, a poor drug metabolizer with a CYP2D6 defect is identified by administering one of the probe drugs, debrisoquine, sparteine or dextromethorphan, then testing urine for the ratio of unmodified to modified drug. Poor metabolizers (PM) exhibit physiologic accumulation of unmodified drug and have a high metabolic ratio of probe drug to metabolite. This bioassay has a number of limitations: lack of patient cooperation, adverse reactions to probe drugs, and inaccuracy due to coadministration of other pharmacological agents or disease effects. Genetic assays by

RFLP (restriction fragment length polymorphism), ASO PCR (allele specific oligonucleotide hybridization to PCR products or PCR using mutant/wildtype specific oligo primers), SSCP (single stranded conformation polymorphism) and TGGE/DGGE (temperature or denaturing gradient gel electrophoresis), MDE (mutation detection electrophoresis) are time-consuming, technically demanding and limited in the number of gene mutation sites that can be tested at one time.

The difficulties inherent in previous methods are overcome by the use of DNA chips to analyze mutations in biotransformation genes. The development of VLSIPS™ technology has provided methods for making very large arrays of oligonucleotide probes in very small areas. See U.S. 5,143,854, WO 90/15070 and WO 92/10092, each of which is incorporated herein by reference.

Microfabricated arrays of large numbers of oligonucleotide probes, called "DNA chips" offer great promise for a wide variety of applications. The present application describes the use of such chips for *inter alia* analysis of polymorphisms and copy number variations in genes of interest, particularly, biotransformation genes, such as cytochromes P450.

SUMMARY OF THE INVENTION

The invention provides methods for determining the copy number of a gene present in an individual. In such methods, a plurality of polymorphic sites from an individual are analyzed and the number of different polymorphic forms present at each site is thereby determined. Gene copy number is then assigned as the highest number of polymorphic forms present at a single site. Typically, the polymorphisms are in the gene whose copy number is being determined or in flanking sequences, although the polymorphism can be present elsewhere provided they are on the same chromosome as the gene whose copy number is being determined. To illustrate, if a single polymorphic form is present at each of the plurality of sites, the copy number of the gene is assigned as 1. If two polymorphic forms are present at one site and a single polymorphic form is present

at each other of the plurality of sites, the copy number of the gene is assigned as 2. If three polymorphic forms are present at a first polymorphic site, a single polymorphic form is present at a second polymorphic site and two polymorphic
5 forms are present at a third polymorphic site and the copy number of the gene is assigned as 3.

Often some or all of the polymorphisms analyzed are silent polymorphisms. Such silent polymorphisms can be present in a noncoding segment of the gene, such as an
10 intronic segment, or in sequences flanking the gene. The more polymorphisms analyzed, the more likely one is to obtain an accurate result. Typically, analysis of about 10 or 50 polymorphisms is sufficient. Nucleic acids for analysis are typically prepared by obtaining a tissue sample from the
15 individual containing the gene and amplifying the gene or a fragment thereof.

Polymorphisms are typically analyzed using probe arrays. Such analysis can be performed by contacting a nucleic acid comprising the gene or a fragment thereof with an array of
20 oligonucleotides, the array comprising a plurality of subarrays, each subarray spanning a polymorphic site and complementarity to at least one polymorphic form of the gene at the site. Hybridization intensities of the nucleic acid to the oligonucleotides in the array are then detected. The
25 pattern of hybridization indicates the number of polymorphic forms present at each polymorphic site. In some methods, subarrays are subdivided into probe groups, with different probe groups comprising probes complementary to different polymorphic forms at a site. In some methods, probe groups
30 are subdivided into two or more probe sets. A first probe set comprises a plurality of probes spanning a polymorphic site of the gene, each probe comprising a segment of at least six nucleotides exactly complementary to a polymorphic form of the gene at the site, the segment including at least one
35 interrogation position complementary to a corresponding nucleotide in the polymorphic form. A second probe set comprises a corresponding probe for each probe in the first probe set, the corresponding probe in the second probe set

being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least six nucleotides thereof that includes the at least one interrogation position, except that the at least one

5 interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets. In some methods, third and fourth probe sets are also present. In such methods, the second, third and fourth probe sets, each comprise a corresponding probe for

10 each probe in the first probe set, the probes in the second, third and fourth probe sets being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least six nucleotides thereof that includes the at least one interrogation position, except that

15 the at least one interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe sets.

Often, the methods also analyze a phenotype-determining polymorphic site in the same gene as the polymorphisms used to

20 determined copy number to determine which polymorphic form(s) are present at the site. This information can be used to diagnoses a phenotype of the patient based on the polymorphic form(s) present at the phenotype-determining polymorphic site.

In some methods, analysis of polymorphisms for

25 determination of copy number and analysis of a phenotype-determining polymorphisms are performed using the same probe array. Such methods entail hybridizing a sample comprising a target nucleic acid comprising one or more alleles of the gene to an array of oligonucleotide probes immobilized on a solid

30 support. Such an array comprises a first probe set comprising a plurality of probes, each probe comprising a segment of at least six nucleotides exactly complementary to a reference form of the gene, the segment including at least one interrogation position complementary to a corresponding

35 nucleotide in the reference form of the gene, the reference form of the gene having a silent polymorphic site and a site of potential mutation associated with a phenotypic change. Such an array also contains a second, and often, third and

fourth probe sets. The second, third and fourth probe sets, each comprise a corresponding probe for each probe in the first probe set, the probes in the second, third and fourth probe sets being identical to a sequence comprising the
5 corresponding probe from the first probe set or a subsequence of at least six nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe
10 sets. The method entails determining which probes, relative to one another, bind to the target nucleic acid, whereby the relative binding of probes having an interrogation position aligned with the silent polymorphism indicates the number of different alleles of the gene in the sample and the relative
15 binding of probes having an interrogation position aligned with the mutation indicates whether the mutation is present in at least one of the alleles.

The invention further provides arrays of probes immobilized on a solid support for analyzing biotransformation
20 genes. In a first embodiment, the invention provides a tiling strategy employing an array of immobilized oligonucleotide probes comprising at least two sets of probes. A first probe set comprises a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to
25 a subsequence of a reference sequence from a biotransformation gene, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence. A second probe set comprises a corresponding probe for each probe in the first probe set, the
30 corresponding probe in the second probe set being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is
35 occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets. The probes in the first probe set have at least two interrogation positions corresponding to two contiguous

nucleotides in the reference sequence. One interrogation position corresponds to one of the contiguous nucleotides, and the other interrogation position to the other. In this, and other forms of array, biotransformation genes of particular interest for analysis include cytochromes P450, particularly 2D6 and 2C19, N-acetyl transferase II, glucose 6-phosphate dehydrogenase, pseudocholinesterase, catechol-O-methyl transferase, and dihydropyridine dehydrogenase.

In a second embodiment, the invention provides a tiling strategy employing an array comprising four probe sets. A first probe set comprises a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of a reference sequence from a biotransformation gene, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence. Second, third and fourth probe sets each comprise a corresponding probe for each probe in the first probe set. The probes in the second, third and fourth probe sets are identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe sets.

In a third embodiment, the invention provides arrays comprising first and second groups of probe sets, each group comprising first, second and optionally, third and fourth probe sets as defined above. The first probe sets in the first and second groups are designed to be exactly complementary to first and second reference sequences. For example, the first reference can include a site of mutation rendering the gene nonfunctional, and the second reference sequence can include a site of a silent polymorphism.

In a fourth embodiment, the invention provides a block of oligonucleotides probes (sometimes referred to as an optiblock) immobilized on a support. The array comprises a perfectly matched probe comprising a segment of at least three

nucleotides exactly complementary to a subsequence of a reference sequence from a biotransformation gene, the segment having a plurality of interrogation positions respectively corresponding to a plurality of nucleotides in the reference sequence. For each interrogation position, the array further comprises three mismatched probes, each identical to a sequence comprising the perfectly matched probe or a subsequence of at least three nucleotides thereof including the plurality of interrogation positions, except in the interrogation position, which is occupied by a different nucleotide in each of the three mismatched probes and the perfectly matched probe.

In a fifth embodiment (sometimes referred to as deletion tiling), the invention provides an array comprising at least four probes. A first probe comprises first and second segments, each of at least three nucleotides and exactly complementary to first and second subsequences of a reference sequence from a biotransformation gene, the segments including at least one interrogation position corresponding to a nucleotide in the reference sequence, wherein either (1) the first and second subsequences are noncontiguous, or (2) the first and second subsequences are contiguous and the first and second segments are inverted relative to the complement of the first and second subsequences in the reference sequence. The array further comprises second, third and fourth probes, identical to a sequence comprising the first probe or a subsequence thereof comprising at least three nucleotides from each of the first and second segments, except in the at least one interrogation position, which differs in each of the probes.

In a sixth embodiment, the invention provides a method of comparing a target nucleic acid with a reference sequence from a biotransformation gene. The method comprises hybridizing a sample comprising the target nucleic acid to one of the arrays of oligonucleotide probes described above. The method then determines which probes, relative to one another, specifically bind to the target nucleic acid, the relative specific binding of corresponding probes indicating whether a

nucleotide in the target sequence is the same or different from the corresponding nucleotide in the reference sequence.

For example, for the array of the second embodiment which has four probe sets, the array can be analyzed by comparing
5 the relative specific binding of four corresponding probes from the first, second, third and fourth probe sets, assigning a nucleotide in the target sequence as the complement of the interrogation position of the probe having the greatest specific binding, and repeating these steps until each
10 nucleotide of interest in the target sequence has been assigned.

In some methods, the reference sequence includes a site of a mutation in the biotransformation gene and a silent polymorphism in or flanking the biotransformation gene, and
15 the target nucleic acid comprises one or more different alleles of the biotransformation gene. In this situation, the relative specific binding of probes having an interrogation position aligned with the silent polymorphism indicates the number of different alleles and the relative
20 specific binding of probes having an interrogation position aligned with the mutation indicates whether the mutation is present in at least one of the alleles.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Basic tiling strategy. The figure illustrates
25 the relationship between an interrogation position (I) and a corresponding nucleotide (n) in the reference sequence, and between a probe from the first probe set and corresponding probes from second, third and fourth probe sets.

Fig. 2: Segment of complementarity in a probe from the
30 first probe set.

Fig. 3A: Incremental succession of probes in a basic tiling strategy. The figure shows four probe sets, each having three probes. Note that each probe differs from its predecessor in the same set by the acquisition of a 5'
35 nucleotide and the loss of a 3' nucleotide, as well as in the nucleotide occupying the interrogation position.

Fig. 3B: Arrangement of probe sets in tiling arrays lacking a perfectly matched probe set.

Fig. 4A: Exemplary arrangement of lanes on a chip. The chip shows four probe sets, each having five probes and each having a total of five interrogation positions (I1-I5), one per probe.

Fig. 4B: A tiling strategy for analyzing closing spaced mutations.

Fig. 4C: A tiling strategy for avoiding loss of signal due to probe self-annealing.

Fig. 5: Hybridization pattern of chip having probes laid down in lanes. Dark patches indicate hybridization. The probes in the lower part of the figure occur at the column of the array indicated by the arrow when the probes length is 15 and the interrogation position 7.

Fig. 6: Strategies for detecting deletion and insertion mutations. Bases in brackets may or may not be present.

Fig. 7: Block tiling strategy. The perfectly matched probe has three interrogation positions. The probes from the other probe sets have only one of these interrogation positions.

Fig. 8: Multiplex tiling strategy. Each probe has two interrogation positions.

Fig. 9: Helper mutation strategy. The segment of complementarity differs from the complement of the reference sequence at a helper mutation as well as the interrogation position.

Fig. 10: Layout of probes on chip for analysis of cytochrome P450 2D6 and cytochrome P450 2C19.

Fig. 11: Alternative tiling for analysis of CYP2D6/CYP2D7 polymorphism.

Fig. 12: Optiblock for analysis of CYP2D6 P34S polymorphism.

Fig. 13: The chip shown in Fig. 10 hybridized to a CYP2D6-B target.

Fig. 14: Magnification of the hybridization patterns of the cytochrome P450 2D6 L421P and S486 polymorphism opti-tiling blocks.

Fig. 15: Hybridization of the chip shown in Fig. 10 to cytochrome P450 2C19.

DETAILED DESCRIPTION OF THE INVENTION

5 The invention provides a number of strategies for comparing a polynucleotide of known sequence (a reference sequence) with variants of that sequence (target sequences). The comparison can be performed at the level of entire genomes, chromosomes, genes, exons or introns, or can focus on
10 individual mutant sites and immediately adjacent bases. The strategies allow detection of variations, such as mutations or polymorphisms, in the target sequence irrespective whether a particular variant has previously been characterized. The strategies both define the nature of a variant and identify
15 its location in a target sequence.

 The strategies employ arrays of oligonucleotide probes immobilized to a solid support. Target sequences are analyzed by determining the extent of hybridization at particular probes in the array. The strategy in selection of probes
20 facilitates distinction between perfectly matched probes and probes showing single-base or other degrees of mismatches. The strategy usually entails sampling each nucleotide of interest in a target sequence several times, thereby achieving a high degree of confidence in its identity. This level of
25 confidence is further increased by sampling of adjacent nucleotides in the target sequence to nucleotides of interest. The present tiling strategies result in sequencing and comparison methods suitable for routine large-scale practice with a high degree of confidence in the sequence output.

30 I. GENERAL TILING STRATEGIES

A. Selection of Reference Sequence

 The chips are designed to contain probes exhibiting complementarity to one or more selected reference sequence whose sequence is known. The chips are used to read a target
35 sequence comprising either the reference sequence itself or variants of that sequence. Target sequences may differ from the reference sequence at one or more positions but show a

high overall degree of sequence identity with the reference sequence (e.g., at least 75, 90, 95, 99, 99.9 or 99.99%). Any polynucleotide of known sequence can be selected as a reference sequence. Reference sequences of interest include

5 sequences known to include mutations or polymorphisms associated with phenotypic changes having clinical significance in human patients. For example, the CFTR gene and P53 gene in humans have been identified as the location of several mutations resulting in cystic fibrosis or cancer

10 respectively. Other reference sequences of interest include those that serve to identify pathogenic microorganisms and/or are the site of mutations by which such microorganisms acquire drug resistance (e.g., the HIV reverse transcriptase gene). Other reference sequences of interest include regions where

15 polymorphic variations are known to occur (e.g., the D-loop region of mitochondrial DNA). These reference sequences have utility for, e.g., forensic or epidemiological studies. Other reference sequences of interest include p34 (related to p53), p65 (implicated in breast, prostate and liver cancer), and DNA

20 segments encoding cytochromes P450 and other biotransformation genes (see Meyer et al., *Pharmac. Ther.* 46, 349-355 (1990)). Other reference sequences of interest include HLA classes I and II. Other reference sequences of interest include those from the genome of pathogenic viruses (e.g., hepatitis (A, B,

25 or C), herpes virus (e.g., VZV, HSV-1, HAV-6, HSV-II, and CMV, Epstein Barr virus), adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia

30 virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus and arboviral encephalitis virus. Other reference sequences of interest are from genomes or episomes of pathogenic bacteria, particularly regions that confer drug resistance or allow phylogenetic

35 characterization of the host (e.g., 16S rRNA or corresponding DNA). For example, such bacteria include chlamydia, rickettsial bacteria, mycobacteria, staphylococci, treptocci, pneumonococci, meningococci and conococci, klebsiella,

proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lyme disease bacteria. Other reference sequences of interest include those in which

5 mutations result in the following autosomal recessive disorders: sickle cell anemia, β -thalassemia, phenylketonuria, galactosemia, Wilson's disease, hemochromatosis, severe combined immunodeficiency, alpha-1-antitrypsin deficiency, albinism, alkaptonuria, lysosomal

10 storage diseases and Ehlers-Danlos syndrome. Other reference sequences of interest include those in which mutations result in X-linked recessive disorders: hemophilia, glucose-6-phosphate dehydrogenase, agammaglobulinemia, diabetes insipidus, Lesch-Nyhan syndrome, muscular dystrophy, Wiskott-

15 Aldrich syndrome, Fabry's disease and fragile X-syndrome. Other reference sequences of interest includes those in which mutations result in the following autosomal dominant disorders: familial hypercholesterolemia, polycystic kidney disease, Huntingdon's disease, hereditary spherocytosis,

20 Marfan's syndrome, von Willebrand's disease, neurofibromatosis, tuberous sclerosis, hereditary hemorrhagic telangiectasia, familial colonic polyposis, Ehlers-Danlos syndrome, myotonic dystrophy, muscular dystrophy, osteogenesis imperfecta, acute intermittent porphyria, and von Hippel-

25 Lindau disease.

The length of a reference sequence can vary widely from a full-length genome, to an individual chromosome, episome, gene, component of a gene, such as an exon, intron or regulatory sequences, to a few nucleotides. A reference

30 sequence of between about 2, 5, 10, 20, 50, 100, 5000, 1000, 5,000 or 10,000, 20,000 or 100,000 nucleotides is common. Sometimes only particular regions of a sequence (e.g., exons of a gene) are of interest. In such situations, the particular regions can be considered as separate reference

35 sequences or can be considered as components of a single reference sequence, as matter of arbitrary choice.

A reference sequence can be any naturally occurring, mutant, consensus or purely hypothetical sequence of

nucleotides, RNA or DNA. For example, sequences can be obtained from computer data bases, publications or can be determined or conceived *de novo*. Usually, a reference sequence is selected to show a high degree of sequence identity to envisaged target sequences. Often, particularly, where a significant degree of divergence is anticipated between target sequences, more than one reference sequence is selected. Combinations of wildtype and mutant reference sequences are employed in several applications of the tiling strategy.

B. Chip Design

1. Basic Tiling Strategy

The basic tiling strategy provides an array of immobilized probes for analysis of target sequences showing a high degree of sequence identity to one or more selected reference sequences. The strategy is first illustrated for an array that is subdivided into four probe sets, although it will be apparent that in some situations, satisfactory results are obtained from only two probe sets. A first probe set comprises a plurality of probes exhibiting perfect complementarity with a selected reference sequence. The perfect complementarity usually exists throughout the length of the probe. However, probes having a segment or segments of perfect complementarity that is/are flanked by leading or trailing sequences lacking complementarity to the reference sequence can also be used. Within a segment of complementarity, each probe in the first probe set has at least one interrogation position that corresponds to a nucleotide in the reference sequence. That is, the interrogation position is aligned with the corresponding nucleotide in the reference sequence, when the probe and reference sequence are aligned to maximize complementarity between the two. If a probe has more than one interrogation position, each corresponds with a respective nucleotide in the reference sequence. The identity of an interrogation position and corresponding nucleotide in a particular probe in the first probe set cannot be determined simply by inspection of the probe in the first set. As will become apparent, an

interrogation position and corresponding nucleotide is defined by the comparative structures of probes in the first probe set and corresponding probes from additional probe sets.

In principle, a probe could have an interrogation
5 position at each position in the segment complementary to the reference sequence. Sometimes, interrogation positions provide more accurate data when located away from the ends of a segment of complementarity. Thus, typically a probe having a segment of complementarity of length x does not contain more
10 than $x-2$ interrogation positions. Since probes are typically 9-21 nucleotides, and usually all of a probe is complementary, a probe typically has 1-19 interrogation positions. Often the probes contain a single interrogation position, at or near the center of probe.

15 For each probe in the first set, there are, for purposes of the present illustration, up to three corresponding probes from three additional probe sets. See Fig. 1. Thus, there are four probes corresponding to each nucleotide of interest in the reference sequence. Each of the
20 four corresponding probes has an interrogation position aligned with that nucleotide of interest. Usually, the probes from the three additional probe sets are identical to the corresponding probe from the first probe set with one exception. The exception is that at least one (and often only
25 one) interrogation position, which occurs in the same position in each of the four corresponding probes from the four probe sets, is occupied by a different nucleotide in the four probe sets. For example, for an A nucleotide in the reference sequence, the corresponding probe from the first probe set has
30 its interrogation position occupied by a T, and the corresponding probes from the additional three probe sets have their respective interrogation positions occupied by A, C, or G, a different nucleotide in each probe. Of course, if a probe from the first probe set comprises trailing or flanking
35 sequences lacking complementarity to the reference sequences (see Fig. 2), these sequences need not be present in corresponding probes from the three additional sets. Likewise corresponding probes from the three additional sets can

contain leading or trailing sequences outside the segment of complementarity that are not present in the corresponding probe from the first probe set. Occasionally, the probes from the additional three probe set are identical (with the exception of interrogation position(s)) to a contiguous subsequence of the full complementary segment of the corresponding probe from the first probe set. In this case, the subsequence includes the interrogation position and usually differs from the full-length probe only in the omission of one or both terminal nucleotides from the termini of a segment of complementarity. That is, if a probe from the first probe set has a segment of complementarity of length n , corresponding probes from the other sets will usually include a subsequence of the segment of at least length $n-2$. Thus, the subsequence is usually at least 3, 4, 7, 9, 15, 21, or 25 nucleotides long, most typically, in the range of 9-21 nucleotides. The subsequence should be sufficiently long to allow a probe to hybridize detectably more strongly to a variant of the reference sequence mutated at the interrogation position than to the reference sequence.

The probes can be oligodeoxyribonucleotides or oligoribonucleotides, or any modified forms of these polymers that are capable of hybridizing with a target nucleic sequence by complementary base-pairing. Complementary base pairing means sequence-specific base pairing which includes *e.g.*, Watson-Crick base pairing as well as other forms of base pairing such as Hoogsteen base pairing. Modified forms include 2'-O-methyl oligoribonucleotides and so-called PNAs, in which oligodeoxyribonucleotides are linked via peptide bonds rather than phosphodiester bonds. The probes can be attached by any linkage to a support (*e.g.*, 3', 5' or via the base). 3' attachment is more usual as this orientation is compatible with the preferred chemistry for solid phase synthesis of oligonucleotides.

The number of probes in the first probe set (and as a consequence the number of probes in additional probe sets) depends on the length of the reference sequence, the number of nucleotides of interest in the reference sequence and the

number of interrogation positions per probe. In general, each nucleotide of interest in the reference sequence requires the same interrogation position in the four sets of probes.

Consider, as an example, a reference sequence of 100

5 nucleotides, 50 of which are of interest, and probes each having a single interrogation position. In this situation, the first probe set requires fifty probes, each having one interrogation position corresponding to a nucleotide of
10 fourth probe sets each have a corresponding probe for each probe in the first probe set, and so each also contains a total of fifty probes. The identity of each nucleotide of interest in the reference sequence is determined by comparing the relative hybridization signals at four probes having
15 interrogation positions corresponding to that nucleotide from the four probe sets.

In some reference sequences, every nucleotide is of interest. In other reference sequences, only certain portions in which variants (e.g., mutations or polymorphisms) are
20 concentrated are of interest. In other reference sequences, only particular mutations or polymorphisms and immediately adjacent nucleotides are of interest. Usually, the first probe set has interrogation positions selected to correspond to at least a nucleotide (e.g., representing a point mutation)
25 and one immediately adjacent nucleotide. Usually, the probes in the first set have interrogation positions corresponding to at least 3, 10, 50, 100, 1000, or 20,000 contiguous nucleotides. The probes usually have interrogation positions corresponding to at least 5, 10, 30, 50, 75, 90, 99 or
30 sometimes 100% of the nucleotides in a reference sequence. Frequently, the probes in the first probe set completely span the reference sequence and overlap with one another relative to the reference sequence. For example, in one common arrangement each probe in the first probe set differs from
35 another probe in that set by the omission of a 3' base complementary to the reference sequence and the acquisition of a 5' base complementary to the reference sequence. See Figure 3A.

The number of probes on the chip can be quite large (e.g., 10^5 - 10^6). However, often only a relatively small proportion (i.e., less than about 50%, 25%, 10%, 5% or 1%) of the total number of probes of a given length are selected to pursue a particular tiling strategy. For example, a complete set of octomer probes comprises 65,536 probes; thus, an array of the invention typically has fewer than 32,768 octomer probes. A complete array of decamer probes comprises 1,048,576 probes; thus, an array of the invention typically has fewer than about 500,000 decamer probes. Often arrays have a lower limit of 25, 50 or 100 probes and an upper limit of 1,000,000, 100,000, 10,000 or 1000 probes. The arrays can have other components besides the probes such as linkers attaching the probes to a support.

Some advantages of the use of only a proportion of all possible probes of a given length include: (i) each position in the array is highly informative, whether or not hybridization occurs; (ii) nonspecific hybridization is minimized; (iii) it is straightforward to correlate hybridization differences with sequence differences, particularly with reference to the hybridization pattern of a known standard; and (iv) the ability to address each probe independently during synthesis, using high resolution photolithography, allows the array to be designed and optimized for any sequence. For example the length of any probe can be varied independently of the others.

For conceptual simplicity, the probes in a set are usually arranged in order of the sequence in a lane across the chip. A lane contains a series of overlapping probes, which represent or tile across, the selected reference sequence (see Figure 3A). The components of the four sets of probes are usually laid down in four parallel lanes, collectively constituting a row in the horizontal direction and a series of 4-member columns in the vertical direction. Corresponding probes from the four probe sets (i.e., complementary to the same subsequence of the reference sequence) occupy a column. Each probe in a lane usually differs from its predecessor in the lane by the omission of a base at one end and the

inclusion of additional base at the other end as shown in Figure 3A. However, this orderly progression of probes can be interrupted by the inclusion of control probes or omission of probes in certain columns of the array. Such columns serve as
5 controls to orient the chip, or gauge the background, which can include target sequence nonspecifically bound to the chip.

The probes sets are usually laid down in lanes such that all probes having an interrogation position occupied by an A form an A-lane, all probes having an interrogation
10 position occupied by a C form a C-lane, all probes having an interrogation position occupied by a G form a G-lane, and all probes having an interrogation position occupied by a T (or U) form a T lane (or a U lane). Note that in this arrangement there is not a unique correspondence between probe sets and
15 lanes. Thus, the probe from the first probe set is laid down in the A-lane, C-lane, A-lane, A-lane and T-lane for the five columns in Figure 4A. The interrogation position on a column of probes corresponds to the position in the target sequence whose identity is determined from analysis of hybridization to
20 the probes in that column. Thus, I_1 - I_5 respectively correspond to N_1 - N_5 in Figure 4A. The interrogation position can be anywhere in a probe but is usually at or near the central position of the probe to maximize differential hybridization signals between a perfect match and a single-
25 base mismatch. For example, for an 11 mer probe, the central position is the sixth nucleotide.

Although the array of probes is usually laid down in rows and columns as described above, such a physical arrangement of probes on the chip is not essential. Provided
30 that the spatial location of each probe in an array is known, the data from the probes can be collected and processed to yield the sequence of a target irrespective of the physical arrangement of the probes on a chip. In processing the data, the hybridization signals from the respective probes can be
35 reassorted into any conceptual array desired for subsequent data reduction whatever the physical arrangement of probes on the chip.

A range of lengths of probes can be employed in the chips. As noted above, a probe may consist exclusively of a complementary segments, or may have one or more complementary segments juxtaposed by flanking, trailing and/or intervening segments. In the latter situation, the total length of complementary segment(s) is more important than the length of the probe. In functional terms, the complementary segment(s) of the first probe sets should be sufficiently long to allow the probe to hybridize detectably more strongly to a reference sequence compared with a variant of the reference including a single base mutation at the nucleotide corresponding to the interrogation position of the probe. Similarly, the complementary segment(s) in corresponding probes from additional probe sets should be sufficiently long to allow a probe to hybridize detectably more strongly to a variant of the reference sequence having a single nucleotide substitution at the interrogation position relative to the reference sequence. A probe usually has a single complementary segment having a length of at least 3 nucleotides, and more usually at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or 30 bases exhibiting perfect complementarity (other than possibly at the interrogation position(s) depending on the probe set) to the reference sequence. In bridging strategies, where more than one segment of complementarity is present, each segment provides at least three complementary nucleotides to the reference sequence and the combined segments provide at least two segments of three or a total of six complementary nucleotides. As in the other strategies, the combined length of complementary segments is typically from 6-30 nucleotides, and preferably from about 9-21 nucleotides. The two segments are often approximately the same length. Often, the probes (or segment of complementarity within probes) have an odd number of bases, so that an interrogation position can occur in the exact center of the probe.

In some chips, all probes are the same length. Other chips employ different groups of probe sets, in which case the probes are of the same size within a group, but

differ between different groups. For example, some chips have one group comprising four sets of probes as described above in which all the probes are 11 mers, together with a second group comprising four sets of probes in which all of the probes are
5 13 mers. Of course, additional groups of probes can be added. Thus, some chips contain, e.g., four groups of probes having sizes of 11 mers, 13 mers, 15 mers and 17 mers. Other chips have different size probes within the same group of four probe sets. In these chips, the probes in the first set can vary in
10 length independently of each other. Probes in the other sets are usually the same length as the probe occupying the same column from the first set. However, occasionally different lengths of probes can be included at the same column position in the four lanes. The different length probes are included
15 to equalize hybridization signals from probes irrespective of whether A-T or C-G bonds are formed at the interrogation position.

The length of probe can be important in distinguishing between a perfectly matched probe and probes
20 showing a single-base mismatch with the target sequence. The discrimination is usually greater for short probes. Shorter probes are usually also less susceptible to formation of secondary structures. However, the absolute amount of target sequence bound, and hence the signal, is greater for larger
25 probes. The probe length representing the optimum compromise between these competing considerations may vary depending on *inter alia* the GC content of a particular region of the target DNA sequence, secondary structure, synthesis efficiency and cross-hybridization. In some regions of the target, depending
30 on hybridization conditions, short probes (e.g., 11 mers) may provide information that is inaccessible from longer probes (e.g., 19 mers) and vice versa. Maximum sequence information can be read by including several groups of different sized probes on the chip as noted above. However, for many regions
35 of the target sequence, such a strategy provides redundant information in that the same sequence is read multiple times from the different groups of probes. Equivalent information can be obtained from a single group of different sized probes

in which the sizes are selected to maximize readable sequence at particular regions of the target sequence. The strategy of customizing probe length within a single group of probe sets minimizes the total number of probes required to read a particular target sequence. This leaves ample capacity for the chip to include probes to other reference sequences.

The invention provides an optimization block which allows systematic variation of probe length and interrogation position to optimize the selection of probes for analyzing a particular nucleotide in a reference sequence. The block comprises alternating columns of probes complementary to the wildtype target and probes complementary to a specific mutation. The interrogation position is varied between columns and probe length is varied down a column. Hybridization of the chip to the reference sequence or the mutant form of the reference sequence identifies the probe length and interrogation position providing the greatest differential hybridization signal.

Variation of interrogation position in probes for analyzing different regions of a target sequence offers a number of advantages. If a segment of a target sequence contains two closely spaced mutations, m1, and m2, and probes for analyzing that segment have an interrogation position at or near the middle, then no probe has an interrogation position aligned with one of the mutations without overlapping the other mutation (see first probe in Figure 4B). Thus, the presence of a mutation would have to be detected by comparing the hybridization signal of a single-mismatched probe with a double-mismatched probe. By contrast, if the interrogation position is near the 3' end of the probes, probes can have their interrogation position aligned with m1 without overlapping m2 (second probe in Figure 4B). Thus, the mutation can be detected by a comparison of a perfectly matched probe with single based mismatched probes. Similarly, if the interrogation position is near the 5' end of the probes, probes can have their interrogation position aligned with m2 without overlapping m1 (third probe in Figure 4B).

Variation of the interrogation position also offers the advantage of reducing loss of signal due to self-annealing of certain probes. Figure 4C shows a target sequence having a nucleotide X, which can be read either from the relative
5 signals of the four probes having a central interrogation position (shown at the left of the figure) or from the four probes having the interrogation position near the three prime end (shown at the right of the figure). Only the probes having the central interrogation position are capable of self-
10 annealing. Thus, a higher signal is obtained from the probes having the interrogation position near the terminus.

The probes are designed to be complementary to either strand of the reference sequence (e.g., coding or non-coding). Some chips contain separate groups of probes, one
15 complementary to the coding strand, the other complementary to the noncoding strand. Independent analysis of coding and noncoding strands provides largely redundant information. However, the regions of ambiguity in reading the coding strand are not always the same as those in reading the noncoding
20 strand. Thus, combination of the information from coding and noncoding strands increases the overall accuracy of sequencing.

Some chips contain additional probes or groups of probes designed to be complementary to a second reference
25 sequence. The second reference sequence is often a subsequence of the first reference sequence bearing one or more commonly occurring mutations or interstrain variations. The second group of probes is designed by the same principles as described above except that the probes exhibit
30 complementarity to the second reference sequence. The inclusion of a second group is particularly useful for analyzing short subsequences of the primary reference sequence in which multiple mutations are expected to occur within a short distance commensurate with the length of the probes (i.e., two
35 or more mutations within 9 to 21 bases). Of course, the same principle can be extended to provide chips containing groups of probes for any number of reference sequences. Alternatively, the chips may contain additional probe(s) that

do not form part of a tiled array as noted above, but rather serves as probe(s) for a conventional reverse dot blot. For example, the presence of mutation can be detected from binding of a target sequence to a single oligomeric probe harboring the mutation. Preferably, an additional probe containing the equivalent region of the wildtype sequence is included as a control.

Although only a subset of probes is required to analyze a particular target sequence, it is quite possible that other probes superfluous to the contemplated analysis are also included on the chip. In the extreme case, the chip could contain a complete set of all probes of a given length notwithstanding that only a small subset is required to analyze the particular reference sequence of interest. Although such a situation might appear wasteful of resources, a chip including a complete set of probes offers the advantage of including the appropriate subset of probes for analyzing any reference sequence. Such a chip also allows simultaneous analysis of a reference sequence from different subsets of probes (e.g., subsets having the interrogation site at different positions in the probe).

In its simplest terms, the analysis of a chip reveals whether the target sequence is the same or different from the reference sequence. If the two are the same, all probes in the first probe set show a stronger hybridization signal than corresponding probes from other probe sets. If the two are different, most probes from the first probe set still show a stronger hybridization signal than corresponding probes from the other probe sets, but some probes from the first probe set do not. Thus, when a probe from another probe set light up more strongly than the corresponding probe from the first probe set, this provides a simple visual indication that the target sequence and reference sequence differ.

The chips also reveal the nature and position of differences between the target and reference sequence. The chips are read by comparing the intensities of labelled target bound to the probes in an array. Specifically, for each nucleotide of interest in the target sequence, a comparison is

performed between probes having an interrogation position aligned with that position. These probes form a column (actual or conceptual) on the chip. For example, a column often contains one probe from each of A, C, G and T lanes.

5 The nucleotide in the target sequence is identified as the complement of the nucleotide occupying the interrogation position in the probe showing the highest hybridization signal from a column. Fig. 6 shows the hybridization pattern of a chip hybridized to its reference sequence. The dark square in

10 each column represents the probe from the column having the highest hybridization signal. The sequence can be read by following the pattern of dark squares from left to right across the chip. The first dark square is in the A lane indicating that the nucleotide occupying the interrogation

15 position of the probe represented by this square is an A. The first nucleotide in the reference sequence is the complement of nucleotide occupying the interrogation position of this probe (i.e., a T). Similarly, the second dark square is in the T-lane, from which it can be deduced that the second

20 nucleotide in the reference sequence is an A. Likewise the third dark square is in the T-lane, from which it can be deduced that the third nucleotide in the reference sequence is also an A, and so forth. By including probes in the first probe set (and by implication in the other probe sets) with

25 interrogation positions corresponding to every nucleotide in a reference sequence, it is possible to read substantially every nucleotide in a target sequence, thereby revealing the complete or nearly complete sequence of the target.

Of the four probes in a column, only one can

30 exhibit a perfect match to the target sequence whereas the others usually exhibit at least a one base pair mismatch. The probe exhibiting a perfect match usually produces a substantially greater hybridization signal than the other three probes in the column and is thereby easily identified.

35 However, in some regions of the target sequence, the distinction between a perfect match and a one-base mismatch is less clear. Thus, a call ratio is established to define the ratio of signal from the best hybridizing probes to the second

best hybridizing probe that must be exceeded for a particular target position to be read from the probes. A high call ratio ensures that few if any errors are made in calling target nucleotides, but can result in some nucleotides being scored
5 as ambiguous, which could in fact be accurately read. A lower call ratio results in fewer ambiguous calls, but can result in more erroneous calls. It has been found that at a call ratio of 1.2 virtually all calls are accurate. However, a small but significant number of bases (e.g., up to about 10%) may have
10 to be scored as ambiguous.

Although small regions of the target sequence can sometimes be ambiguous, these regions usually occur at the same or similar segments in different target sequences. Thus, for precharacterized mutations, it is known in advance whether
15 that mutation is likely to occur within a region of unambiguously determinable sequence.

An array of probes is most useful for analyzing the reference sequence from which the probes were designed and variants of that sequence exhibiting substantial sequence
20 similarity with the reference sequence (e.g., several single-base mutants spaced over the reference sequence). When an array is used to analyze the exact reference sequence from which it was designed, one probe exhibits a perfect match to the reference sequence, and the other three probes in the same
25 column exhibits single-base mismatches. Thus, discrimination between hybridization signals is usually high and accurate sequence is obtained. High accuracy is also obtained when an array is used for analyzing a target sequence comprising a variant of the reference sequence that has a single mutation
30 relative to the reference sequence, or several widely spaced mutations relative to the reference sequence. At different mutant loci, one probe exhibits a perfect match to the target, and the other three probes occupying the same column exhibit single-base mismatches, the difference (with respect to
35 analysis of the reference sequence) being the lane in which the perfect match occurs.

For target sequences showing a high degree of divergence from the reference strain or incorporating several

closely spaced mutations from the reference strain, a single group of probes (i.e., designed with respect to a single reference sequence) will not always provide accurate sequence for the highly variant region of this sequence. At some particular columnar positions, it may be that no single probe exhibits perfect complementarity to the target and that any comparison must be based on different degrees of mismatch between the four probes. Such a comparison does not always allow the target nucleotide corresponding to that columnar position to be called. Deletions in target sequences can be detected by loss of signal from probes having interrogation positions encompassed by the deletion. However, signal may also be lost from probes having interrogation positions closely proximal to the deletion resulting in some regions of the target sequence that cannot be read. Target sequence bearing insertions will also exhibit short regions including and proximal to the insertion that usually cannot be read.

The presence of short regions of difficult-to-read target because of closely spaced mutations, insertions or deletions, does not prevent determination of the remaining sequence of the target as different regions of a target sequence are determined independently. Moreover, such ambiguities as might result from analysis of diverse variants with a single group of probes can be avoided by including multiple groups of probe sets on a chip. For example, one group of probes can be designed based on a full-length reference sequence, and the other groups on subsequences of the reference sequence incorporating frequently occurring mutations or strain variations.

A particular advantage of the present sequencing strategy over conventional sequencing methods is the capacity simultaneously to detect and quantify proportions of multiple target sequences. Such capacity is valuable, e.g., for diagnosis of patients who are heterozygous with respect to a gene or who are infected with a virus, such as HIV, which is usually present in several polymorphic forms. Such capacity is also useful in analyzing targets from biopsies of tumor cells and surrounding tissues. The presence of multiple

target sequences is detected from the relative signals of the four probes at the array columns corresponding to the target nucleotides at which diversity occurs. The relative signals of the four probes for the mixture under test are compared
5 with the corresponding signals from a homogeneous reference sequence. An increase in a signal from a probe that is mismatched with respect to the reference sequence, and a corresponding decrease in the signal from the probe which is matched with the reference sequence, signal the presence of a
10 mutant strain in the mixture. The extent in shift in hybridization signals of the probes is related to the proportion of a target sequence in the mixture. Shifts in relative hybridization signals can be quantitatively related to proportions of reference and mutant sequence by prior
15 calibration of the chip with seeded mixtures of the mutant and reference sequences. By this means, a chip can be used to detect variant or mutant strains constituting as little as 1, 5, 20, or 25 % of a mixture of stains.

Similar principles allow the simultaneous analysis
20 of multiple target sequences even when none is identical to the reference sequence. For example, with a mixture of two target sequences bearing first and second mutations, there would be a variation in the hybridization patterns of probes having interrogation positions corresponding to the first and
25 second mutations relative to the hybridization pattern with the reference sequence. At each position, one of the probes having a mismatched interrogation position relative to the reference sequence would show an increase in hybridization signal, and the probe having a matched interrogation position
30 relative to the reference sequence would show a decrease in hybridization signal. Analysis of the hybridization pattern of the mixture of mutant target sequences, preferably in comparison with the hybridization pattern of the reference sequence, indicates the presence of two mutant target
35 sequences, the position and nature of the mutation in each strain, and the relative proportions of each strain.

In a variation of the above method, several target sequences target sequences are differentially labelled before

being simultaneously applied to the array. For example, each different target sequence can be labelled with a fluorescent labels emitting at different wavelength. After applying a mixtures of target sequence to the arrays, the individual
5 target sequences can be distinguished and independently analyzed by virtue of the differential labels. For example, the methods target sequences obtained from a patient at different stages of a disease can be differently labelled and analyzed simultaneously, facilitating identification of new
10 mutations.

2. Omission of Probes

The basic strategy outlined above employs four probes to read each nucleotide of interest in a target sequence. One probe (from the first probe set) shows a
15 perfect match to the reference sequence and the other three probes (from the second, third and fourth probe sets) exhibit a mismatch with the reference sequence and a perfect match with a target sequence bearing a mutation at the nucleotide of interest. The provision of three probes from the second,
20 third and fourth probe sets allows detection of each of the three possible nucleotide substitutions of any nucleotide of interest. However, in some reference sequences or regions of reference sequences, it is known in advance that only certain mutations are likely to occur. Thus, for example, at one site
25 it might be known that an A nucleotide in the reference sequence may exist as a T mutant in some target sequences but is unlikely to exist as a C or G mutant. Accordingly, for analysis of this region of the reference sequence, one might include only the first and second probe sets, the first probe
30 set exhibiting perfect complementarity to the reference sequence, and the second probe set having an interrogation position occupied by an invariant A residue (for detecting the T mutant). In other situations, one might include the first, second and third probes sets (but not the fourth) for
35 detection of a wildtype nucleotide in the reference sequence and two mutant variants thereof in target sequences. In some chips, probes that would detect silent mutations (i.e., not affecting amino acid sequence) are omitted.

Some chips effectively contain the second, third and optionally, the fourth probes sets described in the basic tiling strategy (i.e., the mismatched probe sets) but omit some or all of the probes from the first probe set (i.e., perfectly matched probes). Therefore, such chips comprise at least two probe sets, which will arbitrarily be referred to as probe sets A and B (to avoid confusion with the nomenclature used to describe the four probe sets in the basic tiling strategy). Probe set A has a plurality of probes. Each probe comprises a segment exactly complementary to a subsequence of a reference sequence except in at least one interrogation position. The interrogation position corresponds to a nucleotide in the reference sequence juxtaposed with the interrogation position when the reference sequence and probe are maximally aligned. Probe set B has a corresponding probe for each probe in the first probe set. The corresponding probe in probe set B is identical to a sequence comprising the corresponding probe from the first probe set or a subsequence thereof that includes the at least one (and usually only one) interrogation position except that the at least one interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the probe sets A and B. An additional probe set C, if present, also comprises a corresponding probe for each probe in the probe set A except in the at least one interrogation position, which differs in the corresponding probes from probe sets A, B and C. The arrangement of probe sets A, B and C is shown in Figure 3B. Figure 3B is the same as Figure 3A except that the first probe set has been omitted and the second, third and fourth probe sets in Figure 3A have been relabelled as probe sets A, B and C in Figure 3B.

Chips lacking perfectly matched probes are preferably analyzed by hybridization to both target and reference sequences. The hybridizations can be performed sequentially, or, if the target and reference are differentially labelled, concurrently. The hybridization data are then analyzed in two ways. First, considering only the hybridization signals of the probes to the target sequence,

one compares the signals of corresponding probes for each position of interest in the target sequence. For a position of mismatch with the reference sequence, one of the probes having an interrogation position aligned with that position in the target sequence shows a substantially higher signal than other corresponding probes. The nucleotide occupying the position of mismatch in the target sequence is the complement of the nucleotide occupying the interrogation position of the corresponding probe showing the highest signal. For a position where target and reference sequence are the same, none of the corresponding probes having an interrogation position aligned with that position in the target sequence is matched, and corresponding probes generally show weak signals, which may vary somewhat from each other.

In a second level of analysis, the ratio of hybridization signals to the target and reference sequences is determined for each probe in the array. For most probes in the array the ratio of hybridization signals is about the same. For such a probe, it can be deduced that the interrogation position of the probe corresponds to a nucleotide that is the same in target and reference sequences. A few probes show a much higher ratio of target hybridization to reference hybridization than the majority of probes. For such a probe, it can be deduced that the interrogation position of the probe corresponds to a nucleotide that differs between target and reference sequences, and that in the target, this nucleotide is the complement of the nucleotide occupying the interrogation position of the probe. The second level of analysis serves as a control to confirm the identification of differences between target and reference sequence from the first level of analysis.

3. Wildtype Probe Lane

When the chips comprise four probe sets, as discussed *supra*, and the probe sets are laid down in four lanes, an A lane, a C-lane, a G lane and a T or U lane, the probe having a segment exhibiting perfect complementarity to a reference sequence varies between the four lanes from one column to another. This does not present any significant

difficulty in computer analysis of the data from the chip. However, visual inspection of the hybridization pattern of the chip is sometimes facilitated by provision of an extra lane of probes, in which each probe has a segment exhibiting perfect
5 complementarity to the reference sequence. See Figure 4A. This extra lane of probes is called the wildtype lane and contains only probes from the first probe set. Each wildtype lane probe has a segment that is identical to a segment from one of the probes in the other four lanes (which lane
10 depending on the column position). The wildtype lane hybridizes to a target sequence at all nucleotide positions except those in which deviations from the reference sequence occurs. The hybridization pattern of the wildtype lane thereby provides a simple visual indication of mutations.

15 **4. Deletion, Insertion and Multiple-Mutation Probes**

Some chips provide an additional probe set specifically designed for analyzing deletion mutations. The additional probe set comprises a probe corresponding to each
20 probe in the first probe set as described above. However, a probe from the additional probe set differs from the corresponding probe in the first probe set in that the nucleotide occupying the interrogation position is deleted in the probe from the additional probe set. See Fig. 6.
25 Optionally, the probe from the additional probe set bears an additional nucleotide at one of its termini relative to the corresponding probe from the first probe set (shown in brackets in Fig. 6). The probe from the additional probe set will hybridize more strongly than the corresponding probe from
30 the first probe set to a target sequence having a single base deletion at the nucleotide corresponding to the interrogation position. Additional probe sets are provided in which not only the interrogation position, but also an adjacent nucleotide is deleted.

35 Similarly, other chips provide additional probe sets for analyzing insertions. For example, one additional probe set has a probe corresponding to each probe in the first probe set as described above. However, the probe in the additional

probe set has an extra T nucleotide inserted adjacent to the interrogation position. See Fig. 6 (the extra T is shown in a square box). Optionally, the probe has one fewer nucleotide at one of its termini relative to the corresponding probe from the first probe set (shown in brackets). The probe from the additional probe set hybridizes more strongly than the corresponding probe from the first probe set to a target sequence having an A insertion to the left of nucleotide "n" the reference sequence in Fig. 6. Similar additional probe sets can be constructed having C, G or A nucleotides inserted adjacent to the interrogation position.

Usually, four such additional probe sets, one for each nucleotide, are used in combination. Comparison of the hybridization signal of the probes from the additional probe sets with the corresponding probe from the first probe set indicates whether the target sequence contains an insertion. For example, if a probe from one of the additional probe sets shows a higher hybridization signal than a corresponding probe from the first probe set, it is deduced that the target sequence contains an insertion adjacent to the corresponding nucleotide (n) in the target sequence. The inserted base in the target is the complement of the inserted base in the probe from the additional probe set showing the highest hybridization signal. If the corresponding probe from the first probe set shows a higher hybridization signal than the corresponding probes from the additional probe sets, then the target sequence does not contain an insertion to the left of corresponding position ("n" in Fig. 6) in the target sequence.

Other chips provide additional probes (multiple-mutation probes) for analyzing target sequences having multiple closely spaced mutations. A multiple-mutation probe is usually identical to a corresponding probe from the first set as described above, except in the base occupying the interrogation position, and except at one or more additional positions, corresponding to nucleotides in which substitution may occur in the reference sequence. The one or more additional positions in the multiple mutation probe are

occupied by nucleotides complementary to the nucleotides occupying corresponding positions in the reference sequence when the possible substitutions have occurred.

5. Block Tiling

5 In block tiling, a perfectly matched (or wildtype) probe is compared with multiple sets of mismatched or mutant probes. The perfectly matched probe and the multiple sets of mismatched probes with which it is compared collectively form a group or block of probes on the chip. Each set comprises at
10 least one, and usually, three mismatched probes. Fig. 7 shows a perfectly matched probe (CAATCGA) having three interrogation positions (I_1 , I_2 and I_3). The perfectly matched probe is compared with three sets of probes (arbitrarily designated A, B and C), each having three mismatched probes. In set A, the
15 three mismatched probes are identical to a sequence comprising the perfectly matched probe or a subsequence thereof including the interrogation positions, except at the first interrogation position. That is, the mismatched probes in the set A differ from the perfectly matched probe set at the first
20 interrogation position. Thus, the relative hybridization signals of the perfectly matched probe and the mismatched probes in the set A indicates the identity of the nucleotide in a target sequence corresponding to the first interrogation position. This nucleotide is the complement of the nucleotide
25 occupying the interrogation position of the probe showing the highest signal. Similarly, set B comprises three mismatched probes, that differ from the perfectly matched probe at the second interrogation position. The relative hybridization intensities of the perfectly matched probe and the three
30 mismatched probes of set B reveal the identity of the nucleotide in the target sequence corresponding to the second interrogation position (i.e., n_2 in Fig. 7). Similarly, the three mismatched probes in set C in Fig. 7 differ from the perfectly matched probe at the third interrogation position.
35 Comparison of the hybridization intensities of the perfectly matched probe and the mismatched probes in the set C reveals the identity of the nucleotide in the target sequence corresponding to the third interrogation position (n_3).

As noted above, a perfectly matched probe may have seven or more interrogation positions. If there are seven interrogation positions, there are seven sets of three mismatched probe, each set serving to identify the nucleotide
5 corresponding to one of the seven interrogation positions. Similarly, if there are 20 interrogation positions in the perfectly matched probe, then 20 sets of three mismatched probes are employed. As in other tiling strategies, selected probes can be omitted if it is known in advance that only
10 certain types of mutations are likely to arise.

Each block of probes allows short regions of a target sequence to be read. For example, for a block of probes having seven interrogation positions, seven nucleotides in the target sequence can be read. Of course, a chip can
15 contain any number of blocks depending on how many nucleotides of the target are of interest. The hybridization signals for each block can be analyzed independently of any other block. The block tiling strategy can also be combined with other tiling strategies, with different parts of the same reference
20 sequence being tiled by different strategies.

The block tiling strategy is a species of the basic tiling strategy discussed above, in which the probe from the first probe set has more than one interrogation position. The perfectly matched probe in the block tiling strategy is
25 equivalent to a probe from the first probe set in the basic tiling strategy. The three mismatched probes in set A in block tiling are equivalent to probes from the second, third and fourth probe sets in the basic tiling strategy. The three mismatched probes in set B of block tiling are equivalent to
30 probes from additional probe sets in basic tiling arbitrarily designated the fifth, sixth and seventh probe sets. The three mismatched probes in set C of blocking tiling are equivalent to probes from three further probe sets in basic tiling arbitrarily designated the eighth, ninth and tenth probe sets.

35 The block tiling strategy offers two advantages over a basic strategy in which each probe in the first set has a single interrogation position. One advantage is that the same sequence information can be obtained from fewer probes. A

second advantage is that each of the probes constituting a block (i.e., a probe from the first probe set and a corresponding probe from each of the other probe sets) can have identical 3' and 5' sequences, with the variation
5 confined to a central segment containing the interrogation positions. The identity of 3' sequence between different probes simplifies the strategy for solid phase synthesis of the probes on the chip and results in more uniform deposition of the different probes on the chip, thereby in turn
10 increasing the uniformity of signal to noise ratio for different regions of the chip.

6. Multiplex Tiling

In the block tiling strategy discussed above, the identity of a nucleotide in a target or reference sequence is
15 determined by comparison of hybridization patterns of one probe having a segment showing a perfect match with that of other probes (usually three other probes) showing a single base mismatch. In multiplex tiling, the identity of at least two nucleotides in a reference or target sequence is
20 determined by comparison of hybridization signal intensities of four probes, two of which have a segment showing perfect complementarity or a single base mismatch to the reference sequence, and two of which have a segment showing perfect complementarity or a double-base mismatch to a segment. The
25 four probes whose hybridization patterns are to be compared each have a segment that is exactly complementary to a reference sequence except at two interrogation positions, in which the segment may or may not be complementary to the reference sequence. The interrogation positions correspond to
30 the nucleotides in a reference or target sequence which are determined by the comparison of intensities. The nucleotides occupying the interrogation positions in the four probes are selected according to the following rule. The first
35 interrogation position is occupied by a different nucleotide in each of the four probes. The second interrogation position is also occupied by a different nucleotide in each of the four probes. In two of the four probes, designated the first and second probes, the segment is exactly complementary to the

reference sequence except at not more than one of the two
interrogation positions. In other words, one of the
interrogation positions is occupied by a nucleotide that is
complementary to the corresponding nucleotide from the
5 reference sequence and the other interrogation position may or
may not be so occupied. In the other two of the four probes,
designated the third and fourth probes, the segment is exactly
complementary to the reference sequence except that both
interrogation positions are occupied by nucleotides which are
10 noncomplementary to the respective corresponding nucleotides
in the reference sequence.

There are number of ways of satisfying these
conditions depending on whether the two nucleotides in the
reference sequence corresponding to the two interrogation
15 positions are the same or different. If these two nucleotides
are different in the reference sequence (probability 3/4), the
conditions are satisfied by each of the two interrogation
positions being occupied by the same nucleotide in any given
probe. For example, in the first probe, the two interrogation
20 positions would both be A, in the second probe, both would be
C, in the third probe, each would be G, and in the fourth
probe each would be T or U. If the two nucleotides in the
reference sequence corresponding to the two interrogation
positions are different, the conditions noted above are
25 satisfied by each of the interrogation positions in any one of
the four probes being occupied by complementary nucleotides.
For example, in the first probe, the interrogation positions
could be occupied by A and T, in the second probe by C and G,
in the third probe by G and C, and in the fourth probe, by T and
30 A. See (Fig. 8).

When the four probes are hybridized to a target that
is the same as the reference sequence or differs from the
reference sequence at one (but not both) of the interrogation
positions, two of the four probes show a double-mismatch with
35 the target and two probes show a single mismatch. The
identity of probes showing these different degrees of mismatch
can be determined from the different hybridization signals.
From the identity of the probes showing the different degrees

of mismatch, the nucleotides occupying both of the interrogation positions in the target sequence can be deduced.

For ease of illustration, the multiplex strategy has been initially described for the situation where there are two
5 nucleotides of interest in a reference sequence and only four probes in an array. Of course, the strategy can be extended to analyze any number of nucleotides in a target sequence by using additional probes. In one variation, each pair of
10 interrogation positions is read from a unique group of four probes. In a block variation, different groups of four probes exhibit the same segment of complementarity with the reference sequence, but the interrogation positions move within a block. The block and standard multiplex tiling variants can of course be used in combination for different regions of a reference
15 sequence. Either or both variants can also be used in combination with any of the other tiling strategies described.

7. Helper Mutations

Occasionally, small regions of a reference sequence give a low hybridization signal as a result of annealing of
20 probes. The self-annealing reduces the amount of probe effectively available for hybridizing to the target. Although such regions of the target are generally small and the reduction of hybridization signal is usually not so substantial as to obscure the sequence of this region, this
25 concern can be avoided by the use of probes incorporating helper mutations. A helper mutation refers to a position of mismatch in a probe other than at an interrogation position. The helper mutation(s) serve to break-up regions of internal complementarity within a probe and thereby prevent annealing.
30 Usually, one or two helper mutations are quite sufficient for this purpose. The inclusion of helper mutations can be beneficial in any of the tiling strategies noted above. In general each probe having a particular interrogation position has the same helper mutation(s). Thus, such probes have a
35 segment in common which shows perfect complementarity with a reference sequence, except that the segment contains at least one helper mutation (the same in each of the probes) and at least one interrogation position (different in all of the

probes). For example, in the basic tiling strategy, a probe from the first probe set comprises a segment containing an interrogation position and showing perfect complementarity with a reference sequence except for one or two helper mutations. The corresponding probes from the second, third and fourth probe sets usually comprise the same segment (or sometimes a subsequence thereof including the helper mutation(s) and interrogation position), except that the base occupying the interrogation position varies in each probe.

See Fig. 9.

Usually, the helper mutation tiling strategy is used in conjunction with one of the tiling strategies described above. The probes containing helper mutations are used to tile regions of a reference sequence otherwise giving low hybridization signal (e.g., because of self-complementarity), and the alternative tiling strategy is used to tile intervening regions.

8. Bridging Strategy

Probes that contain partial matches to two separate (i.e., non contiguous) subsequences of a target sequence sometimes hybridize strongly to the target sequence. In certain instances, such probes have generated stronger signals than probes of the same length which are perfect matches to the target sequence. It is believed (but not necessary to the invention) that this observation results from interactions of a single target sequence with two or more probes simultaneously. This invention exploits this observation to provide arrays of probes having at least first and second segments, which are respectively complementary to first and second subsequences of a reference sequence. Optionally, the probes may have a third or more complementary segments. These probes can be employed in any of the strategies noted above. The two segments of such a probe can be complementary to disjoint subsequences of the reference sequences or contiguous subsequences. If the latter, the two segments in the probe are inverted relative to the order of the complement of the reference sequence. The two subsequences of the reference

sequence each typically comprises about 3 to 30 contiguous nucleotides. The subsequences of the reference sequence are sometimes separated by 0, 1, 2 or 3 bases. Often the sequences, are adjacent and nonoverlapping.

5 For example, a wildtype probe is created by complementing two sections of a reference sequence (indicated by subscript and superscript) and reversing their order. The interrogation position is designated (*) and is apparent from comparison of the structure of the wildtype probe with the
10 three mismatched probes. The corresponding nucleotide in the reference sequence is the "a" in the superscripted segment.

Reference: 5' T_{GGCTA}^{CGAGG}AATCATCTGTTA

*

15 Probes: 3' GCTCC CCGAT (Probe from first probe set)
3' GCACC CCGAT
3' GCCCC CCGAT
3' GCGCC CCGAT

The expected hybridizations are:

20 Match: GCTCCCCGAT
...TGGCTACGAGGAATCATCTGTTA
GCTCCCCGAT

Mismatch:
25 GCTCCCCGAT
...TGGCTACGAGGAATCATCTGTTA
GCGCCCCGAT

Bridge tilings are specified using a notation which gives the length of the two constituent segments and the relative position of the interrogation position. The
30 designation n/m indicates a segment complementary to a region of the reference sequence which extends for n bases and is located such that the interrogation position is in the mth base from the 5' end. If m is larger than n, this indicates that the entire segment is to the 5' side of the interrogation
35 position. If m is negative, it indicates that the interrogation position is the absolute value of m bases 5' of the first base of the segment (m cannot be zero). Probes comprising multiple segments, such as n/m + a/b + ... have a first segment at the 3' end of the probe and additional

segments added 5' with respect to the first segment. For example, a 4/8 tiling consists of (from the 3' end of the probe) a 4 base complementary segment, starting 7 bases 5' of the interrogation position, followed by a 6 base region in which the interrogation position is located at the third base. Between these two segments, one base from the reference sequence is omitted. By this notation, the set shown above is a 5/3 + 5/8 tiling. Many different tilings are possible with this method, since the lengths of both segments can be varied, as well as their relative position (they may be in either order and there may be a gap between them) and their location relative to the interrogation position.

As an example, a 16 mer oligo target was hybridized to a chip containing all 4¹⁰ probes of length 10. The chip includes short tilings of both standard and bridging types. The data from a standard 10/5 tiling was compared to data from a 5/3 + 5/8 bridge tiling (see Table 1). Probe intensities (mean count/pixel) are displayed along with discrimination ratios (correct probe intensity / highest incorrect probe intensity). Missing intensity values are less than 50 counts. Note that for each base displayed the bridge tiling has a higher discrimination value.

TABLE 1: Comparison of Standard and Bridge Tilings

TILING	PROBE BASE:		CORRECT PROBE BASE			
			C	A	C	C
STANDARD (10/5)	A		92	496	294	299
	C		536	148	532	534
		G		69	167	72 52
	T		146	95	212	126
DISCRIMINATION:				3.7	3.0	1.8 1.8
BRIDGING 5/3 + 5/8	A		-	404	-	156
	C		276	-	345	379
	G		-	80	-	-
	T		-	-	-	58
DISCRIMINATION:				>5.5	5.1	2.4 1.26

The bridging strategy offers the following advantages:

(1) Higher discrimination between matched and mismatched probes,

(2) The possibility of using longer probes in a bridging tiling, thereby increasing the specificity of the
5 hybridization, without sacrificing discrimination,

(3) The use of probes in which an interrogation position is located very off-center relative to the regions of target complementarity. This may be of particular advantage when, for example, when a probe centered about one region of the
10 target gives low hybridization signal. The low signal is overcome by using a probe centered about an adjoining region giving a higher hybridization signal.

(4) Disruption of secondary structure that might result in annealing of certain probes (see previous discussion of
15 helper mutations).

9. Deletion Tiling

Deletion tiling is related to both the bridging and helper mutant strategies described above. In the deletion strategy, comparisons are performed between probes sharing a
20 common deletion but differing from each other at an interrogation position located outside the deletion. For example, a first probe comprises first and second segments, each exactly complementary to respective first and second subsequences of a reference sequence, wherein the first and
25 second subsequences of the reference sequence are separated by a short distance (e.g., 1 or 2 nucleotides). The order of the first and second segments in the probe is usually the same as that of the complement to the first and second subsequences in the reference sequence. The interrogation position is usually
30 separated from The comparison is performed with three other probes, which are identical to the first probe except at an interrogation position, which is different in each probe.

Reference: . . . AGTACCAGATCTCTAA . . .

Probe set: CATGGNC AGAGA (N = interrogation position).

35 Such tilings sometimes offer superior discrimination in hybridization intensities between the probe having an interrogation position complementary to the target and other probes. Thermodynamically, the difference between the

hybridizations to matched and mismatched targets for the probe set shown above is the difference between a single-base bulge, and a large asymmetric loop (e.g., two bases of target, one of probe). This often results in a larger difference in

5 stability than the comparison of a perfectly matched probe with a probe showing a single base mismatch in the basic tiling strategy.

The superior discrimination offered by deletion tiling is illustrated by Table 2, which compares hybridization

10 data from a standard 10/5 tiling with a (4/8 + 6/3) deletion tiling of the reference sequence. (The numerators indicate the length of the segments and the denominators, the spacing of the deletion from the far termini of the segments.) Probe intensities (mean count/pixel) are displayed along with

15 discrimination ratios (correct probe intensity / highest incorrect probe intensity). Note that for each base displayed the deletion tiling has a higher discrimination value than either standard tiling shown.

TABLE 2. Comparison of Standard and Deletion Tilings

20	TILING	PROBE BASE:	CORRECT PROBE BASE			
			C	A	C	C
25	STANDARD (10/5)	A	92	496	294	299
		C	536	148	532	534
		G	69	167	72	52
		T	146	95	212	126
	DISCRIMINATION:			3.7	3.0	1.8 1.8
30	DELETION 4/8 + 6/3	A	6	412	29	48
		C	297	32	465	160
		G	8	77	10	4
		T	8	26	31	5
	DISCRIMINATION:			37.1	5.4	15 3.3
35	STANDARD (10/7)	A	347	533	228	277
		C	729	194	536	496
		G	232	231	102	89
		T	344	133	163	150
	DISCRIMINATION:			2.1	2.3	2.3 1.8

The use of deletion or bridging probes is quite general. These probes can be used in any of the tiling strategies of the invention. As well as offering superior discrimination, the use of deletion or bridging strategies is advantageous for certain probes to avoid self-hybridization (either within a probe or between two probes of the same sequence).

10. Nucleotide Repeats

Recently a new form of human mutation, expansion of trinucleotide repeats, has been found to cause the diseases of fragile X-syndrome, spinal and bulbar atrophy, myotonic dystrophy and Huntington's disease. See Ross et al., *TINS* 16, 254-259 (1993). Long lengths of trinucleotide repeats are associated with the mutant form of a gene. The longer the length, the more severe the consequences of the mutation and the earlier the age of onset. The invention provides arrays and methods for analyzing the length of such repeats.

The different probes in such an array comprise different numbers of repeats of the complement of the trinucleotide repeat of interest. For example, one probe might be a trimer, having one copy of the repeat, a second probe might be a sixmer, having two copies of the repeat, and a third probe might be a ninmer having three copies, and so forth. The largest probes can have up to about sixty bases or 20 trinucleotide repeats.

The hybridization signal of such probes to a target of trinucleotide repeats is related to the length of the target. It has been found that on increasing the target size up to about the length of the probe, the hybridization signal shows a relatively large increase for each complete trinucleotide repeat unit in the target, and a small increase for each additional base in the target that does not complete a trinucleotide repeat. Thus, for example, the hybridization signals for different target sizes to a 20 mer probe show small increases as the target size is increased from 6-8 nucleotides and a larger increase as the target size is increased to 9 nucleotides.

Arrays of probes having different numbers of repeats are usually calibrated using known amounts of target of different length. For each target of known length, the hybridization intensity is recorded for each probe. Thus, each target size is defined by the relative hybridization signals of a series of probes of different lengths. The array is then hybridized to an unknown target sequence and the relative hybridization signals of the different sized probes are determined. Comparison of the relative hybridization intensity profile for different probes with comparable data for targets of known size allows interpolation of the size of the unknown target. Optionally, hybridization of the unknown target is performed simultaneously with hybridization of a target of known size labelled with a different color.

15 **C. Preparation of Target Samples**

The target polynucleotide, whose sequence is to be determined, is usually isolated from a tissue sample. If the target is genomic, the sample may be from any tissue (except exclusively red blood cells). For example, whole blood, peripheral blood lymphocytes or PBMC, skin, hair or semen are convenient sources of clinical samples. These sources are also suitable if the target is RNA. Blood and other body fluids are also a convenient source for isolating viral nucleic acids. If the target is mRNA, the sample is obtained from a tissue in which the mRNA is expressed. If the polynucleotide in the sample is RNA, it is usually reverse transcribed to DNA. DNA samples or cDNA resulting from reverse transcription are usually amplified, e.g., by PCR. Depending on the selection of primers and amplifying enzyme(s), the amplification product can be RNA or DNA. Paired primers are selected to flank the borders of a target polynucleotide of interest. More than one target can be simultaneously amplified by multiplex PCR in which multiple paired primers are employed. The target can be labelled at one or more nucleotides during or after amplification. For some target polynucleotides (depending on size of sample), e.g., episomal DNA, sufficient DNA is present in the tissue sample to dispense with the amplification step.

When the target strand is prepared in single-stranded form as in preparation of target RNA, the sense of the strand should of course be complementary to that of the probes on the chip. This is achieved by appropriate selection of primers.

- 5 The target is preferably fragmented before application to the chip to reduce or eliminate the formation of secondary structures in the target. The average size of targets segments following hybridization is usually larger than the size of probe on the chip.

10 II. Biotransformation Gene Chips

A. Biotransformation Genes

- Biotransformation genes tiled by the invention include any of the 481 known cytochrome P450 genes, particularly, the human P450 genes (see Nebert, *DNA & Cell Biol.* 10, 1-14
 15 (1991); Nelson et al., *Pharmacogenetics* 6, 1-42 (1996), acetylase genes, monoamine oxidase genes, and genes known to specifically biotransform particular drugs, such as the gene encoding glucuronidase that participates in the pathway by which codeine or morphine are converted to active form. Paul
 20 et al., *J. Pharm. Exp. Ther.* 251, 477 (1989). Other genes of particular interest include P450 2D6, P450 2C19, N-acetyl transferase II, glucose 6-phosphate dehydrogenase, pseudocholinesterase, catechol-O-methyl transferase, thiopurine methyltransferase and dihydropyridine
 25 dehydrogenase. cDNA and at least partial genomic DNA sequences are available for these genes, e.g., from data bases such as GenBank and EMBL (see Table 3).

TABLE 3: ACCESSION NUMBER CYP LIST

GENE	ACCESSION NUMBER(S)	IMPORTANCE
CYP1A1	D12525	Cancer Susceptibility
	D01198	
CYP1A2	M31664	

TABLE 3: ACCESSION NUMBER CYP LIST
(continued)

GENE	ACCESSION NUMBER (S)	IMPORTANCE
	M31665	
	M31666	
	M31667	
	U02993	
CYP2A	X13897	
CYP2A3	M33318	Coumarin 7-hydroxylation
	M33316	
CYP2A4	X13930	
CYP2C8	X54807	
	X54808	
CYP2C9	M61855	Warfarin Metabolism
	J05326	
	M61857	
	J05326	
	L16877	
CYP2C17	M61858	
	J05326	
CYP2C18	M61853	Drug Metabolism
	J05326	
	M61856	
CYP2C19	L07093	S-mephenytoin 4-hydroxylase
	M61854	

TABLE 3: ACCESSION NUMBER CYP LIST
(continued)

GENE	ACCESSION NUMBER (S)	IMPORTANCE
	J05326	
	M15331	
CYP2D6	M20403	Debrisoquin/Sparteine Polymorphism
	M19697	
	M24499	
	X16866	
	X58467	CYP2D7P pseudogene
	X58468	CYP2D8P pseudogene
CYP2E1	D10014	Ethanol Inducible
	J02843	
CYP3A4	D11131	Polymorphic Drug Metabolism
	M14096	
CYP4F2	U02388	Leukotriene B4 omega hydroxylase
NAT2	U23052	Drug Acetylation/Drug Induced Disease
	U23434	
TPMT	U11424	Thiopurine Methyl Transferase-transplantation and childhood leukemia
	U12387	

5

10

Additional genomic sequence flanking the regions already sequenced are easily determined by PCR-based gene walking. See Parker et al., *Nucl. Acids Res.* 19:3055-3060. A specific primer for the sequenced region is primed with a general primer that hybridizes to the flanking region.

The CYP2D6 enzyme has debrisoquine oxidase activity. See e.g., Kimura et al., *Am. J. Human. Genet.* 45, 889-904 (1989).

Several therapeutically important compounds are metabolized by CYP2D6. The list includes cardioactive drugs: 5 β -blockers (bufuralol, propranolol, metoprolol, timolol) and antiarrhythmics (sparteine, encainide, flecainide, mexiletine) (Buchert & Woosley, *Pharmacogenetics* 2, 2-11 (1992); Birgersdotter et al., *Brit. J. Clin. Pharmacol.* 33, 275-280 (1992)); psychoactive drugs including tricyclic 10 antidepressants (imipramine, desipramine, nortriptyline) and antipsychotics (clozapine and haloperidol) (Dahl & Bertilsson, *Pharmacogenetics* 3, 61-70 (1993); Fischer et al., *J. Pharmacol. Exp. Ther.* 260, 1355-1360 (1992); Lerena et al., *Drug Monitor* 14, 92-97 (1992)); as well as a variety of other 15 commonly used drugs including codeine and dextromethorphan (Eichelbaum & Gross, *Pharmac. Ther.* 46, 377-394 (1990)) as well as amphetamine, and cocaine. Ten percent of the general population is defective in P450 2D6, an enzyme that demethylates codeine at an earlier stage in the activation 20 pathway, and therefore derives no analgesic benefit from codeine (see Sindrup & Brosen, *Pharmacogenetics* 5, 335-346 (1995)).

At least seven different polymorphic variants of the CYP2D6 gene demonstrating autosomal recessive inheritance are 25 associated with a poor drug metabolizer phenotype (see Table 4). These alleles are designated CYP2D6A, CYP2D6B, CYP2D6C, CYP2D6D, CYP2D6E, CYP2D6F, and CYP2D6J (Gonzales & Idle, *Clin. Pharmacokinet.* 26(1), 59-70 (1994); Nelson et al., *DNA & Cell Biol.* 12(1), 1-51 (1993)). CYP2D6A, CYP2D6E and CYP2D6F are 30 minor variants of the wild type gene. CYP2D6A has a single nucleotide deletion in exon 5 with a consequent frame shift (Kagimoto et al., *J. Biol. Chem.* 265, 17209-17214 (1990)). CYP2D6E and CYP2D6F are rare, recently described variants (Gonzales & Idle, *supra*). CYP2D6B accounts for about 70% of 35 defective alleles. This variant has point mutations in exons 1, 3, 8 and 9 as well as a base change at the third intron splice site that results in aberrant transcript splicing (Gonzales et al., *Nature* 331, 442-446 (1988); Kagimoto et al.,

J. Biol. Chem. 265, 17209-17214 (1990)). CYP2D6C has a three base deletion in exon 5 (Broly and Meyer, *Pharmacogenetics* 3, 123-130 (1993)) and, on the CYP2D6D allele, the entire functional gene is deleted although the pseudogenes remain
5 intact (Gaedigk et al., *Am. J. Hum. Genet.* 48, 943-950 (1991)). The CYP2D6J allele has base changes in both the first and ninth exons that result in amino acid changes (Yokota et al., *Pharmacogenetics* 3, 256-263 (1993)). The CYP2D6 gene clusters with other CYP2D genes on human
10 chromosome 22. Also present in this region are two or three highly conserved pseudogenes. Of these, CYP2D7P (three variant forms) and CYP2D8P have been isolated and sequenced (Kimura et al., *supra*; Helm & Meyer, *supra*).

TABLE 4

ALLELE	(EXON) NUCLEOTIDE CHANGES	XBAI HAPLOTYPE	ENZYME ACTIVITY	REF
CYP2D6-wt		29 kb	NORMAL	(97) (9)
CYP2D6-LI	(3) 1726 G → C (6) 2938 C → T/296 Arg → Cys (9) 4268 G → C/486 Ser → Thr (6) 2938 C → T/296 Arg → Cys (6) 2938 C → T/296 Arg → Cys	29 kb		(12) (11) (13)
CYP2D6-A	(5) 2637 ΔA	29 kb	ABSENT	(15)
CYP2D6-B	(4) 1934A (+ 6 other mutations)	29 kb 44 kb 9 + 16 kb		(15)
CYP2D6-D	Deletion	11.5 kb (13 kb)		(14)
CYP2D6-E	(6) 3023 A → C/324 His → Pro	29 kb		(99)
CYP2D6-ΔT1795	(3) 1795 ΔT / 152 Try → Gly 153 Stop	29 kb		(98, 100)
CYP2D6-C	(5) 2703-5 ΔAAG / 281 ΔLys	29 kb	DECREASED	(44) (101)
CYP2D6-J	(1) 188 C → T/Pro 34 Pro → Ser (3) 1749 G → C (9) 4268 G → C/486 Ser → Thr	29 kb 44 kb		(16)
CYP2D6-W	(1) 188 C → T/34 Pro → Ser (9) 4268 G → C/486 Ser → Thr	29 kb 44 kb		(102)
CYP2D6-Ch1	(1) 188 C → T/34 Pro → Ser (2) 1127 C → T (3) 1749 G → C (9) 4268 G → C/486 Ser → Thr	29 kb 44 kb		(103)

TABLE 4
(continued)

ALLELE	(EXON) NUCLEOTIDE CHANGES	XBAI HAPLOTYPE	ENZYME ACTIVITY	REF.
(CYP2D6-L) ₁₂	Amplification of D6-L	175 kb	INCREASED	(12)
(CYP2D6-L) ₂	Duplication of D6-L	42 kb		(12)

- 5 Presently used trivial names of CYP2D6 alleles, summary of CYP2D6-Alleles, haplotypes and their phenotypic consequences (modified from U.A. Meyer).
- 9) Kimura et al. Am J Hum Genet 45: 889-904 (1989)
- 11) Armstrong et al. Hum Genet 91: 616-617 (1993)
- 10 12) Johansson et al. PNAS 90: 11825-11829 (1993)
- 13) Tsuneoka et al. J. Biochem Tokyo 114: 263-266 (1993)
- 14) Gaedigk et al. Am J Hum Genet 48: 943-950 (1991)
- 15) Kagimoto et al. J Biol Chem 265: 17209-17214 (1990)
- 16) Yokota et al. Pharmacogenet 3: 256-263 (1993)
- 15 44) Tyndale et al. Pharmacogenet 1: 26-32 (1991)
- 97) Gonzales et al. Nature 331: 442-446 (1988)
- 98) Evert et al. Pharmacogenet 4: 271-274 (1994)
- 99) Evert et al. Naunyn-Schmiedeberg's Arch Pharmacol 350: 434-439 (1994)
- 20 100) Saxena et al. Hum Mol Genet 3: 923-926 (1994)
- 101) Broly et al. Pharmacogenet 3: 123-130 (1993)
- 102) Wang et al. Clin Pharmacol Ther 53: 410-418 (1993)
- 103) Johansson et al. Mol Pharmacol 46: 452-459 (1994)

The 2C19 gene is the principal human determinant of S-mephenytoin hydroxylase. Drugs metabolized by this enzyme in addition to mephenytoin include antidepressants and neuroleptics. Variant alleles are described in de Morais et al., *J. Biol. Chem.* 269(22), 15419-15422 (1994); de Morais et al., *Molecular Pharmacology* 46, 594-598 (1994). Mutations are known to occur at nucleotides 636 (G-A) and 681 (G-A) of the coding sequence.

CYP2E1 is responsible for metabolizing several anesthetics including ethanol. CYP2A6 metabolizes nicotine.

TABLE 4
(continued)

CYP2C9 metabolizes warfarin. A table showing other pairs of drugs and cytochromes P450 that either metabolize the drug or are inhibited by it appears below.

TABLE 4
(continued)

TABLE 5

Drug	Class	Cytochrome P450 (CYP) Isoenzyme			Comments
		Metabolizing Enzymes	Inhibited Enzymes		
Roxithromycin	Antibiotic		3A4		Cin Pharm and Ther 1991, 49, 158
Spiramycin	Antibiotic		3A4		Cin Pharm and Ther 1991, 49, 158
Taxol	Antitumor	6a-hydroxylation - 3A			J Pharm Exp Ther 1994, 268, 1160-1165
Tiracizine	Antiarrhythmic	Urethane cleavage - 2D6			Abstracts - 10th Int Symp Mic & Drug Oxid 1994, p 590
Trimipramine	Antidepressant	Hydroxylation - 2D6			Chem Path Pharm 1993, 82, 111-120
Tropisetron	5-HT3 antagonist	5,6,7-hydroxylation - 2D6			Drug Met Disp 1994, 22, 269-274
Zanoterone	Anticancer	Hydroxylation - 3A4/5			Abstracts - 10th Int Symp Mic & Drug Oxid 1994, p 593
Econazole	Antifungal		3A4 > 1A2 > 2C, 2D6		Clin Pharm and Ther 1991, 49, 158 (abstract P11-37)
Ethosuximide	Anticonvulsant	3A			Xenobiotic 1993, 23, 307-315

Drug	Class	Cytochrome P450 (CYP) Isoenzyme			Comments
		Metabolizing Enzymes	Inhibited Enzymes		
Finasteride	5 α -Reductase Inhibitor	3A4			Abstracts - 10th Int Symp Mic & Drug Oxide 1994, p 594
FK 506	Immunosuppressant	3A4 (major), 2D6 (<10%)			Abstracts - 10th Int Symp Mic & Drug Oxid 1994, p 587
Flexeril	Muscle relaxant	N-demethylation - 1A2, 3A4, 2D6 (minor)			Abstracts - 10th Int Symp Mic & Drug Oxid 1994, p 592
Haloperidol	Neuroleptic Agent	3A			Abstracts - 10th Int Symp Mic & Drug Oxid 1994, p 179
Ibuprofen	NSAID	2C8, 2C9, 2C18			Clin Pharmacokinetics 1994, 26, 59-70
Ifosfamide	Anticancer	4-hydroxylation, N-dechloroethylation - 3A4			Biochem Pharmacol 1994, 47, 1157-1163
Itraconazole	Antifungal		3A4 > 1A2 > 2C, 2D6		Clin Pharm and Ther 1991, 49, 158 (abstract PII-37)
Labetalol	Antihypertensive	2D6			Drug Met Disp 1985, 13, 443-448
Ondansetron	5-HT3 antagonist	7, 8-hydroxylation - 3A, 2D6			Drug Met Disp 1994, 22, 269-274
Oxodipine	Antihypertensive	3A4			J Pharm Exp Ther 1992, 261, 381-386

TABLE 5
(Continued)

Drug	Class	Cytochrome P450 (CYP)		Comments
		Metabolizing Enzymes	Inhibited Enzymes	
Prednisolone	Corticosteroid	3A4		Drug Met Disp 1990, 18, 595-606
Alfentanil	Analgesic	3A4		Anesthesiology 1992, 77, 467-474
Amiflamine	MAO-A Inhibitor	2D6		Clin Pharmacol Ther 1984, 36, 515-519
Azithromycin	Antibiotic	3A4		Clin Pharm and Ther 1991, 49, 158
Benzphetamine	Anorectic	2C8, 2C9, 2C18, 3A4		Clin Pharmacokinetics 1994, 26, 59-70
Captopril	Antihypertensive	2D6		Eur J Clin Pharm 1987, 31, 633-641
Citalopram	Antidepressant	2C18, 2C19		Ther Drug Monit 1993, 15, 11-17
Clarithromycin	Antibiotic	3A4		Clin Pharm and Ther 1991, 49, 158
Clonazepam	Anticonvulsant	Nitroreduction - 3A4		Fundam Clin Pharm 1993, 7, 69-75
Clotrimazole	Antifungal		3A4 > 1A2 > 2C, 2D6	Clin Pharm and Ther 1991, 49, 158 (abstract PII-37)

TABLE 5
(Continued)

Drug	Class	Cytochrome P450 (CYP) Isoenzyme		Comments
		Metabolizing Enzymes	Inhibited Enzymes	
Cocaine		N-demethylation - 3A4		Pharmacol 1993, 46, 294-300
Dapsone	Antibacterial	N-hydroxylation - 3A4		Mol Pharmacol 1992, 41, 975-980
Delavirdine	HIV-1 Reverse transcriptase Inhibitor	Hydroxylation - 3A4 N-dealkylation - 3A4, 2D6		Abstracts - 10th Int Symp Mic & Drug Oxid 1994, p 240
Dextromethorphan	Antitussive	O-demethylation - 2D6 N-demethylation - 3A4, 3A5		Biochem Pharm 1994, 48, 173-182
Diazepam	CNS Depressant	2C8, 2C9, 2C18		Clin Pharmacokinetics 1994, 26, 59-70
Diclofenac	NSAID	2C8, 2C9, 2C18		Clin Pharmacokinetics 1994, 26, 59-70
Tamoxifen	Antiestrogen	3A4, 1A1		ISSX Proceedings Vol. 3, 44
Taxotere	Antimitotic	3A		ISSX Proceedings Vol. 3, 36
Tenoxicam	NSAID	2C		Life Sci 1992, 51, 575-581
Terfenadine	Antihistamine	3A4		Drug Met Disp 1993, 21, 403-409
Timolol	β -blocker	2D6		TIPS 1992, 13, 434-439

TABLE 5
(Continued)

Drug	Class	Cytochrome P450 (CYP) Isoenzyme		Comments
		Metabolizing Enzymes	Inhibited Enzymes	
Thioridazine	Neuroleptic	2D6		TiPS 1992, 13, 434-439
Tolbutamide	Blood glucose lowering agent	2C18		
Tomoxetine	Antidepressant	2D6		TiPS 1992, 13, 434-439
Toremifene	Antiestrogen	3A4/3A5 - (N-demethylation), 1A		ISSX Proceedings Vol 3, 22
Triazolam	Hypnotic	3A4		
Trifluoperidol	Neuroleptic	2D6		TiPS 1992, 13, 434-439
Troleandomycin	Antibiotic		3A4	
Verapamil	Antihypertensive	3A4 (mainly), also 1A2 for D-617 metabolite		Arch Pharmacol 1993, 348, 332-337
Vinblastine	Antitumor	3A4		
Warfarin	Anticoagulant	3A, 2C, 1A2		
Zonisamide	Anticonvulsant	3A		Molec Pharm 1993, 44, 216-221
Acetaminophen	Antipyretic	3A4, 2E1, 1A2		Chem Res Tox 1993, 6, 511

TABLE 5
(Continued)

Drug	Class	Cytochrome P450 (CYP)		Comments
		Metabolizing Enzymes	Inhibited Enzymes	
Amiodarone	Antiarrhythmic	3A - deethylatin, 1A2	1A2, 2C18, 2D6, 3A4	Drug Met Disp 1993, 21, 978-985
Amitriptyline	Antidepressant	2C18, 2D6		
Astemizole	Antihistamine	3A4		
Bufuralol	β -Blocker	2D6		TIPS 1992, 13, 434-439
Carbamazepine	Anticonvulsant	3A		Inducer of 3A4; Clin Pharmacokin 1993, 25, 450-482
Chlorzoxazone	Muscle relaxant	2E1 - 6-OH metabolite		Chem Res Tox 1990, 3, 566-573
Cimetidine	Antiulcer		3A4, 2D6 > 1A2, 2E1	Gastroent 1991, 101, 1680-1691
Ciprofloxacin	Antimicrobial		3A4	Clin Pharmacokin 1992, 23, 132-146
Clomipramine	Antidepressant	2C18, 2F6		
Clozapine	Neuroleptic	2D6		
Codeine	Analgesic	2D6 - demethylation		TIPS 1992, 13, 434-439

TABLE 5
(Continued)

Drug	Class	Cytochrome P450 (CYP) Isoenzyme			Comments
		Metabolizing Enzymes	Inhibited Enzymes		
Cyclosporin	Immunosuppressant	3A4			hydroxylated and N-demethylated metabolites
Dapsone	Antibacterial	3A4			
Debrisoquine	Antihypertensive	2D6			
Desipramine	Antidepressant	2D6			
Dextromethorphan	Antitussive	2D6			TIPS 1992, 13, 434-439
Diazepam	CNS Depressant	2C18			
Diltiazem	Antihypertensive	3A4			
Ebastine	Antihistamine	3A4, 2D6			Structure similar to terfenadine
Encainide	Antiarrhythmic	2D6			
Erythromycin	Antibiotic	3A4			
Felodipine	Antihypertensive	3A4	3A, 1A2		noncompetitive inhibitor
Flecainide	Antiarrhythmic	2D6			

TABLE 5
(Continued)

Drug	Class	Cytochrome P450 (CYP) Isoenzyme		Comments
		Metabolizing Enzymes	Inhibited Enzymes	
Fluoxetine	Antidepressant	2D6, 3A? - Demethylation	2D6 (S>R)	J Pharm Exp Ther 1993, 266, 964-971)
Fluphenazine	Neuroleptic	2D6		TiPS 1992, 13, 434-439
Guanoxan	Antihypertensive	2D6		TiPS 1992, 13, 434-439
Hydrocortisone	Antiinflammatory	3A4		
Imipramine	Antidepressant	2D6-hydroxylation 3A4, 2C19 - demethylation		Biol Pharm Bull 1993, 16, 571
Indoramin	Antihypertensive	2D6		TiPS 1992, 13, 434-439
Ketoconazole	Antifungal		3A4 > 1A2 > 2C, 2D6	
Lidocane	Anesthetic	3A4		Clin Pharm Ther 1989, 46, 521-527
Loratadine	Antihistamine	3A4, 2D6		formation of SCH 34117
Lovastatin	Cholesterol lowering agent	3A4		Arch Biochem Biophys 1991, 290, 355
Mephénytoin	Anticonvulsant	2C18		

TABLE 5
(Continued)

Drug	Class	Cytochrome P450 (CYP) Isoenzyme		Comments
		Metabolizing Enzymes	Inhibited Enzymes	
Metoprolol	Antihypertensive	2D6		
Mexiletine	Antiarrhythmic	2D6		TiPS 1992, 13, 434-439
Nifedipine	Antihypertensive	3A		J Biol Chem 1986, 261, 5051-50601
Nitrendipine	Antihypertensive	3A4		
Nortriptylin	Antidepressant	2D6		TiPS 1992, 13, 434-439
Omeprazole	Antiulcer	3A4 (major), 2C18	2C18	ISSX Proceedings Vol 3, 45-46; Inducer of 1A2, Clin Pharmacokin 1993, 25, 450-482
Perphenazine	Neurologic	2D6		TiPS 1992, 13, 434-439
Phenytoin	Antiepileptic	3A, 2C18		Probable inducer of 3A4; Clin Pharmacokin 1993, 25, 450-482
Propafenone	Antiarrhythmic	3A4, 1A2 - demethylation, 2D6		Molec Pharm 1993, 43, 120-126
Propanalolol	Antihypertensive	2C18, 2D6		

TABLE 5
(Continued)

Drug	Class	Cytochrome P450 (CYP) Isoenzyme		Comments
		Metabolizing Enzymes	Inhibited Enzymes	
Quinidine	Antiarrhythmic	3A4	2D6	
Ranitidine	Antiulcer	2D6		
Bunitrolol	antihypertensive	4-hydroxylation - 2D6		

B. Tissue Sample Preparation

The source of target DNA for detecting mutations in biotransformation genes is usually genomic. In adults, samples can conveniently be obtained from blood or mouthwash or cheek scraping epithelial cells. cDNA can be obtained only from tissues in which biotransformation genes are expressed. The liver is a good source, but a surgical biopsy is required to remove a sample from living patients.

C. Amplification

The target DNA is usually amplified by PCR. Primers can be readily devised from the known genomic and cDNA sequences of biotransformation genes. The selection of primers, of course, depends on the areas of the target sequence that are

to be screened. The choice of primers also depends on the strand to be amplified. Because some nonallelic P450 genes show a high degree of sequence identity, selection of primers can be important in determining whether one or more nonallelic segments is amplified. Usually, primers will be selected to be perfectly complementary to a unique sequence within a selected target resulting in amplification of only that target. Examples of suitable primers are shown in Table 6 (F= forward primer, R= reverse primer).

TABLE 6

SEQUENCE NAME	SEQUENCE
CYP2DE1F	GCCAGGTGTGTCCAGAGGAGCCCAT
CYP2DE1R	CTGGTAGGGGAGCCTCAGCACCTCT
CYP2DE2F	TAGGACTAGGACCTGTAGTCTGGGGT
CYP2DE2R	GGTCCACGGAATCTGTCTCTGT
CYP2DE34F	CTAATGCCTTCATGGCCACGCGCA
CYP2DE34R	TCGGGAGCTCGCCCTGCAGAGA
CYP2DE5F	GGGCCTGAGACTTGTCCAGGTGAA
CYP2DE5R	CCCTCATTCCTCCTGGGACGCTCAA
CYP2DE6F	CCCGTTCTGTCCCGAGTATGCTCT
CYP2DE6R	TCGGCCCCCTGCACTGTTTCCCAGA
CYP2DE7F	GCTGACCCATTGTGGGGACGCAT
CYP2DE7R	CTATCACCAGGTGCTGGTGCTGAGCT
CYP2DE89F	GGGAGACAAACCAGGACCTGCCAGA
CYP2DE89R	CTCAGCCTCAACGTACCCCTGTCT
CYP2D678-F	TGAGAGCAGCTTCAATGATGAGAACCT
CYP2D678-R	GTAGGATCATGAGCAGGAGGCCCA
CYP-PCR8-F	TCCCCCGTGTGTTTGGTGGCA
CYP-PCR9-R	TGCTTTATTGTACATTAGAGC

For analysis of mutants through all or much of a gene, it is often desirable to amplify several segments from several paired primers. The different segments may be amplified sequentially or simultaneously by multiplex PCR. Frequently, fifteen or more segments of a gene are simultaneously

amplified by PCR. The primers and amplification conditions are preferably selected to generate fluorescently labelled DNA targets. Double stranded targets are enzymically degraded to fragments of about 100 bp and denatured before hybridization.

5 **D. Tiling Strategies**

Mutations in biotransformation genes can be detected by any of the tiling strategies noted above. For detection of hitherto uncharacterized mutations, the basic tiling strategy is one suitable strategy. The chips contain probes tiling
10 across some or all of a reference sequence.

For detecting precharacterized mutations, which account for the large majority of poor metabolizers in the preferred reference genes described above, the block tiling strategy is one particularly useful approach. In this strategy, a group
15 (or block) of probes is used to analyze a short segment of contiguous nucleotides (e.g., 3, 5, 7 or 9) from a biotransformation gene centered around the site of a mutation.

In a preferred embodiment, a first group of probes is tiled based on a wildtype reference sequence and a second
20 group is tiled based on a mutant version of the wildtype reference sequence. The mutation can be a point mutation, insertion or deletion or any combination of these. The presence of first and second groups of probes facilitates analysis when multiple target sequences are simultaneously
25 applied to the chip, as is the case when a patient being diagnosed is heterozygous in a biotransformation gene. The principles of chip design and analysis are as described for the CFTR chip.

E. Modifications for Determining Gene Copy Number

30 The tiling arrays of the invention are usually capable of simultaneously analyzing heterozygous alleles of a target sequence. The presence of heterozygous alleles is signalled by two probes having interrogations positions aligned with the mutation showing specific hybridization, rather than one, as
35 would be the case for homozygous alleles. Interpretation of hybridization patterns is, however, sometimes complicated by the presence of less than, or more than, the two expected copies of a biotransformation gene in an individual.

For example, an individual having one wildtype copy of the gene, and a wholly deleted second copy of the gene would show a similar hybridization pattern to an individual with two wildtype copies (other than for possible differences in overall intensity of the pattern). In fact, complete gene deletions of one or both copies of a gene account for approximately 15% of slow metabolizers having defective biotransformation enzymes. Analogous loss of heterozygosity occurs in other diseases such as cancer (p53) and muscular dystrophy (dystrophin gene).

Further, an individual with three wildtype copies of a biotransformation gene would show a similar hybridization pattern to an individual with two copies of the gene, other than for a difference in overall intensity. Individuals having multiple copies of a biotransformation gene are referred to as super metabolizers, because of their elevated levels of enzymes.

Additional complications in interpreting a hybridization pattern can result from the presence of pseudogenes in an individual. A pseudogene is an analog of a true gene that shows strong sequence identity to the true gene but is not expressed. Most pseudogenes having counterparts among the biotransformation genes have been sufficiently well characterized that their presence can be avoided by appropriate selection of amplification primers (i.e., primers are selected that hybridize to the true gene of interest without hybridizing to the pseudogene). For example, 5' TGA GAG CAG CTT CAA TGA TGA GAA CCT 3' and 5' GTA GGA TCA TGA GCA GGA GGC CCC A 3', can be used for amplifying exon 6. However, occasionally a pseudogene might be unexpectedly amplified together with a true gene, and the presence of mutations in the psuedogene (which in fact have no phenotypic effect) might be mistakenly thought to occur in the true gene.

The invention provides tiling arrays to overcome these difficulties by indicating how many copies of a target are present in a sample. In addition to containing the probes required for detecting polymorphism(s) associated with drug sensitive, disease-suscpetibility or other phenotype by the

tiling strategies described above, these arrays contain probes for analyzing polymorphic sites of a target gene, which, in general, do not exert any phenotypic effect (i.e., silent polymorphic sites). The frequency and diversity of such sites is usually greater than that of mutations whose presence does exert a phenotypic effect. Silent sites are predominantly found in intronic regions and in flanking regions (i.e., within about 20 kb of transcribed regions), where selective pressure is generally lower relative to the coding regions. Polymorphisms used to assess gene copy number should be on the same chromosome as the gene containing a phenotype-determining mutation of interest, and are often in the same gene or flanking sequences thereto.

Any number of additional polymorphic sites can be tiled using the same strategies as previously described. For any particular polymorphic site, each form of the polymorphism at that sites serves as a reference sequence for a separate tiling. In some instances, silent polymorphic sites can be amplified from the same primers and on the same amplicon as the sites of potential mutations. In other instances, separate amplification is required.

Silent polymorphic regions can be identified by comparing segments of target DNA, particularly introns and flanking regions, from different individuals. Comparison can be performed using the general tiling strategies disclosed above or by conventional techniques such as single-stranded conformational analysis. See, e.g., Hayashi, *PCR Methods & Applications* 1, 34-38 (1991); Orita, *Proc. Natl. Acad. Sci. USA* 86, 2766-2270 (1989); Orita et al., *Genomics* 5, 874-879 (1989). This method has been successfully employed in dystrophin gene analysis coupled with heteroduplex formation to scan for new mutations. Prior et al., *Human Molecular Genetics* 2, 311-313 (1993).

Analysis of the hybridization pattern of a probe array tiling a silent polymorphic region indicates which of the polymorphic forms are present at this region. Consider a polymorphism constituting a single base change. If the polymorphism and flanking sequences are tiled according to the

basic strategy using four probe sets, there are four probes having an interrogation position aligned with the single base at which the polymorphism occurs. The number of these four probes to show specific hybridization indicates the number of
5 different polymorphic forms present, and hence, the minimum number of copies of a gene present. For example, if two probes show specific hybridization, at least two polymorphic forms are present. There may be more copies of the gene than polymorphic forms observed at any one site, because the same
10 polymorphic form may be present in more than one copy of the gene. However, if sufficient polymorphic sites are examined, it is likely that a site will be found at which each copy of the gene exists in a different polymorphic form. The number of polymorphic sites that needs to be tested depends on the
15 number of polymorphic forms and their relative frequencies at each sites. Typically, the number of sites varies from 1-100, with at least 5, 10, 20 or 50 sites being common. The copy number of a gene can be deduced from the number of polymorphic forms present at the polymorphic site(s) showing the greatest
20 number of polymorphic forms.

If a silent polymorphism is more complicated than a single-base change (e.g., deletion or insertion), the number of polymorphic forms can be determined from alternative tilings to the different forms, as generally described in
25 §I.B.1. For example, if all the perfectly matched probes in a first tiling hybridize, it is concluded that the polymorphic form constituting the reference sequence for the first tiling is present. If, all the perfectly matched probes in two (or more) tilings hybridize, it is concluded that two (or more)
30 polymorphic forms are present.

F. Applications

In general, the biotransformation genes described above are inherited in an autosomal recessive fashion. The presence of a homozygous mutation or two heterozygous mutations in an
35 individual signals that the individual is a poor metabolizer of any drug metabolized by the biotransformation gene in which the mutation occurs. Some individuals with one mutant and one normal gene show a near wildtype phenotype, but other such

individuals show an intermediate phenotype between normal and homozygous mutant. Individuals having additional copies of a biotransformation gene usually express the gene product at higher levels than a wildtype individual.

5 The screening methods can be routinely applied as precaution before administering a drug to a patient for the first time. If the patient is found to lack both copies of a gene expressing an enzyme required for detoxification of a particular drug, the patient generally should not be
10 administered the drug or, should be administered the drug in smaller doses compared with patients having normal levels of the enzyme. The latter course may be necessary if no alternative treatment is available. If the patient is found to lack both copies of a gene expressing an enzyme required
15 for activation of a particular drug, the drug will have no beneficial effect on the patient and should not be administered. Patients having one wildtype copy of a gene and one mutant copy of a gene, and who are at risk of having lower levels of an enzyme, should be administered drugs metabolized
20 by that enzyme only with some caution, again depending on whether alternatives are available. If the drug is detoxified by the enzyme in question, the patient should in general be administered a lower dose of the drug. If the drug is activated by the enzyme, the heterozygous patient should be
25 administered a higher dosage of the drug. The reverse applies for patients having additional copy(ies) of a particular biotransformation gene, who are at risk of having elevated levels of an enzyme. The more rational selection of therapeutic agents that can be made with the benefit of
30 screening results in fewer side effects and greater drug efficacy in poor metabolizer patients.

The methods are also useful for screening populations of patients who are to be used in a clinical trial of a new drug. The screening identifies a pool of patients, each of whom has
35 wildtype levels of the full complement of biotransformation enzymes. The pool of patients are then used for determining safety and efficacy of the drugs. Drugs shown to be effective by such trials are formulated for therapeutic use with a

pharmaceutical carrier such as sterile distilled water, physiological saline, Ringer's solution, dextrose solution, and Hank's solution.

The chips are also useful for screening patients for
5 increased risk of cancer in similar manner to the p53 chips of the invention. Some biotransformation enzymes have roles in activating environmental procarcinogens to carcinogenic form (e.g., 1A1, 2D6, 2E1 and N-acetyltransferase). Mutations in genes encoding these enzymes are associated with reduced
10 cancer risk. Other biotransformation enzymes have roles in detoxifying environmental carcinogens, e.g., glutathione S-transferase M1. Mutations in one, and especially both, copies of genes encoding such enzymes are associated with enhanced susceptibility to cancer. See Shields, *Environmental Health*
15 *Perspectives* 102 (sup. 11), 81-87 (1994).

CYP genotype information can be useful to prevent drug-drug interactions in two main ways. First, some drugs are known to inhibit specific CYP enzymes. When such a drug is given, care should be taken not to give a second drug handled
20 by the inhibited pathway (see Table 4). Second, when a person is genotyped as a poor metabolizer, not only should drug doses be decreased, second drugs handled by the poor metabolizing pathway should not be added to the therapy.

Example

25 **Fig. 10** shows the layout of probes and a computer-simulated hybridization pattern for an exemplary chip containing tilings for CYP2D6 and CYP2C19 (wildtype). The chip contains a number of separate tilings as follows.

(1) A tiling (basic strategy) of all 9 exons plus 5
30 nucleotides of each intron bordering the exons of the CYP2D6 gene. The probes were 14 mers with the interrogation position at nucleotide 7. This tiling is the upper right of the figure (excluding the eleven columns of probe sets on the left of the chip). Each lane of probes is divided into four columns,
35 occupied by probes differing at the interrogation position. At any one column, a nucleotide in the target sequence aligned with the column position is identified as the complement of

the nucleotide in the column having the highest fluorescent intensity.

(2) A tiling (basic strategy) of the complete coding sequence (cDNA/mRNA) of CYP2C19 (wildtype). The probes were 15 mers with the interrogation position at nucleotide 7. This tiling is in the lower half of the figure (excluding the eleven columns of probes at the left of the figure).

(3) A series of "opti-block" tilings for analysis of known mutations in CYP2D6 and CYP2C19. These blocks run down the lefthand eleven columns of the figure. These blocks are labelled 2C19 m2 (mutation in cytochrome P450 2C19), p34S, L91M, H94A, p1085, p1127, Delta T 1795, p1749, ss 1934, G212E, 2637DeltaA, delta281, 296C, H324P, L421P, S486T, 2C19 m1 (mutation in cytochrome P450 2C19). Unless otherwise indicated, the mutations occur in cytochrome P450 2D6.

(4) A series of alternative tilings for analysis of known polymorphic differences between CYP2D6 and its pseudogenes CYP2D7P, CYP2D7AP and CYP2DAP. These tilings are also in the lefthand column of the figure. These tilings are labelled Ex6p 2D6/2D7, Ex2p 2D6/2D7, Ex2p 2D6/2D8, Ex4p 2D6/2D7, Ex4p 2D6/2D8, Ex6p 2D6/2D8, Ex7p 2D6/2D7, and Exp 2D6/2D7.

Fig. 11 shows an alternative tiling designed to distinguish 2D6 from the pseudogene 2D7 in CYP2D6.

Alternative tilings are formed from two interdigitated tilings, each designed according to the basic tiling strategy based on two different reference sequences, in this case 2D6 and 2D7. The first column contains four probes complementary to the CYP2D6 sequence except at the interrogation position. The second column contains four probes complementary to the CYP2D7 sequence except at the interrogation position. The interrogation positions of the first and second columns of probes align with the same positions of the target sequence. The same strategy of alternating probes from the respective 2D6 and 2D7 reference sequences continues throughout the alternative tiling. When the tiling is hybridized to only the CYP2D6 form, only probes complementary to CYP2D6 (i.e., the columns labelled 6) light up. Conversely when the tiling is

hybridized to only the CYP2D7 form, only probes in the columns labelled 7 light up. When the tiling is hybridized to a mixture of CYP2D6 and CYP2D7, the pattern is the sum of the pattern for the two individual forms. The characteristic
5 patterns throughout the tiling allow distinction of whether CYP2D6, CYP2D7 or both are present.

Fig. 12 shows an optiblock of probes for distinguishing the P34S mutation from the wildtype sequence of CYP2D6. In an optiblock, probes are selected based on the block tiling
10 strategy. That is all probes align with the same segment of target DNA but differ in the location of the interrogation position and in whether the probes are tiled based on a wildtype or mutant reference sequence. The notation "n" above the chip indicates that the interrogation position is aligned
15 with the site of the P34S mutation in the target DNA and, the notation n-1 and n + 1 indication interrogation positions aligned one base either side of the site of mutation, and so forth. As in the alternate tiling, probes tiled on wildtype and mutant sequences (sometimes referred to as wildtype and
20 mutant probes) are interdigitated. The result of hybridizing the optiblock to wildtype target is that all columns containing probes tiled based on the wildtype sequence light up. In addition, one column of probes based on the mutant sequence lights up, this being the column of probes having an
25 interrogation position aligned with the "n" nucleotide in the target. The result of hybridizing the optiblock to the mutant target is the reverse; that is all columns of probes tiled based on the mutant target sequence light up, and a single column of probes tiled based on the wildtype sequence lights
30 up. When the optiblock is hybridized to a heterozygous target containing wildtype and mutant forms, the pattern is the sum of those obtained with the individual targets alone. Thus, all three possible targets, homozygous wildtype, homozygous mutant and heterozygote give distinct patterns of
35 hybridization and can be distinguished.

The chip was hybridized with fluorescein-labelled-dGTP double-stranded DNA made by PCR from a plasmid template containing the genomic clone of CYP2D6-B. The entire gene is

amplified as 4 separate PCR products, all of which were present during hybridization. dUTP was incorporated during PCR and the PCR products were treated with uracil DNA glycosylase, then heated to 95°C for 5 min before hybridization to fragment and denature double-stranded material. Hybridization was for 30 min at 37°C in 0.5 M LiCl plus 0.0005% NaLauroylSarkosine. Washing was performed prior to scanning the same solution without target DNA for 5 min at room temperature.

10 **Fig. 13** shows the chip hybridized to a CYP2D6-B target. A portion of the basic tiling pattern is shown magnified in the lower right hand corner. Successive nucleotides in the target sequence can be read by eye by comparing the sequence intensities of the four squares in a column. From top to
15 bottom, these squares are respectively occupied by probes having A, C, G and T at the interrogation position. The nucleotide occupying the position in the target sequence aligned with the interrogation position of a column of probes is the complement of the interrogation position of the probe
20 showing the highest signal. The SS1934 mutation in CYP2D6-B results in a G-A transition and loss of function. The enlarged hybridization pattern in the lower right of the figure has an arrow in the column corresponding to nucleotide 1934. In this column, the probe hybridizing most strongly has
25 a T in the interrogation position. This implies that the corresponding nucleotide in the target is the complement of T, i.e., A, indicating that the mutant form of the target is present. The same result is apparent from the optiblock shown in the upper left of the figure. This block shows three
30 consecutive columns in which the T- probe lights up. Two of these columns are from wildtype and mutant probes having interrogation positions aligned with nucleotide 1934. The third column (the leftmost of the three) is the mutant probe having an interrogation position aligned with nucleotide 1933.
35 **Fig. 14** shows magnifications of the hybridization patterns of L421P and S486 opti-tiling blocks. In each case, the first, third, fifth, sixth, seventh, and ninth columns light up. This pattern indicates that homozygous wildtype

sequence is present (see the idealized pattern for homozygous wildtype in Fig. 12).

In a separate experiment, the chip was hybridized to CYP2C19 cDNA, as shown in Fig. 15. The Figure shows that the lower portion of the chip containing the 2C19 tiles is lit. A magnification of part of the hybridization pattern from the basic tiling sequence is shown in the upper right of the Figure. Again, the sequence can be read by eye by comparing the intensities of the four probes forming a column.

10 III. MODES OF PRACTICING THE INVENTION

A. SYNTHESIS OF ARRAYS

Arrays of probe immobilized on supports can be synthesized by various methods. A preferred method is VLSIPSTM (see Fodor et al., Nature 364, 555-556 (1993); US 5,143,854; EP 476,014; PCT/US94/12305), which entails the use of light to direct the synthesis of oligonucleotide probes in high-density, miniaturized arrays. Algorithms for design of masks to reduce the number of synthesis cycles are described by Hubbel et al., US 5,571,639 and US 5,593,839. Arrays can also be synthesized in a combinatorial fashion by delivering monomers to cells of a support by mechanically constrained flowpaths. See Winkler et al., EP 624,059. Arrays can also be synthesized by spotting monomers reagents on to a support using an ink jet printer. See *id.*; Pease et al., EP 728,520.

25 B. PREPARATION OF LABELED DNA/HYBRIDIZATION TO ARRAY

1. PCR

PCR amplification reactions are typically conducted in a mixture composed of, per reaction: 1 μ l genomic DNA; 10 μ l each primer (10 pmol/ μ l stocks); 10 μ l 10 x PCR buffer (100 mM Tris.Cl pH8.5, 500 mM KCl, 15 mM MgCl₂); 10 μ l 2 mM dNTPs (made from 100 mM dNTP stocks); 2.5 U Taq polymerase (Perkin Elmer AmpliTaqTM, 5 U/ μ l); and H₂O to 100 μ l. The cycling conditions are usually 40 cycles (94°C 45 sec, 55°C 30 sec, 72°C 60 sec) but may need to be varied considerably from sample type to sample type. These conditions are for 0.2 mL thin wall tubes in a Perkin Elmer 9600 thermocycler. See

Perkin Elmer 1992/93 catalogue for 9600 cycle time information. Target, primer length and sequence composition, among other factors, may also affect parameters.

For products in the 200 to 1000 bp size range, check
5 2 μ l of the reaction on a 1.5% 0.5x TBE agarose gel using an appropriate size standard (phiX174 cut with *Hae*III is convenient). The PCR reaction should yield several picomoles of product. It is helpful to include a negative control (i.e., 1 μ l TE instead of genomic DNA) to check for possible
10 contamination. To avoid contamination, keep PCR products from previous experiments away from later reactions, using filter tips as appropriate. Using a set of working solutions and storing master solutions separately is helpful, so long as one does not contaminate the master stock solutions.

15 For simple amplifications of short fragments from genomic DNA it is, in general, unnecessary to optimize Mg^{2+} concentrations. A good procedure is the following: make a master mix minus enzyme; dispense the genomic DNA samples to individual tubes or reaction wells; add enzyme to the master
20 mix; and mix and dispense the master solution to each well, using a new filter tip each time.

2. PURIFICATION

Removal of unincorporated nucleotides and primers from PCR samples can be accomplished using the Promega Magic
25 PCR Preps DNA purification kit. One can purify the whole sample, following the instructions supplied with the kit (proceed from section IIIB, 'Sample preparation for direct purification from PCR reactions'). After elution of the PCR product in 50 μ l of TE or H_2O , one centrifuges the eluate for
30 20 sec at 12,000 rpm in a microfuge and carefully transfers 45 μ l to a new microfuge tube, avoiding any visible pellet. Resin is sometimes carried over during the elution step. This transfer prevents accidental contamination of the linear amplification reaction with 'Magic PCR' resin. Other methods,
35 e.g., size exclusion chromatography, may also be used.

3. LINEAR AMPLIFICATION

In a 0.2 mL thin-wall PCR tube mix: 4 μ l purified PCR product; 2 μ l primer (10 pmol/ μ l); 4 μ l 10 x PCR buffer; 4

μ l dNTPs (2 mM dA, dC, dG, 0.1 mM dT); 4 μ l 0.1 mM dUTP; 1 μ l 1 mM fluorescein dUTP (Amersham RPN 2121); 1 U Taq polymerase (Perkin Elmer, 5 U/ μ l); and add H₂O to 40 μ l. Conduct 40 cycles (92°C 30 sec, 55°C 30 sec, 72°C 90 sec) of PCR. These conditions have been used to amplify a 300 nucleotide mitochondrial DNA fragment but are applicable to other fragments. Even in the absence of a visible product band on an agarose gel, there should still be enough product to give an easily detectable hybridization signal. If one is not treating the DNA with uracil DNA glycosylase (see Section 4), dUTP can be omitted from the reaction.

4. FRAGMENTATION

Purify the linear amplification product using the Promega Magic PCR Preps DNA purification kit, as per Section 2 above. In a 0.2 mL thin-wall PCR tube mix: 40 μ l purified labeled DNA; 4 μ l 10 x PCR buffer; and 0.5 μ l uracil DNA glycosylase (BRL 1U/ μ l). Incubate the mixture 15 min at 37°C, then 10 min at 97°C; store at -20°C until ready to use.

5. HYBRIDIZATION, SCANNING & STRIPPING

A blank scan of the slide in hybridization buffer only is helpful to check that the slide is ready for use. The buffer is removed from the flow cell and replaced with 1 ml of (fragmented) DNA in hybridization buffer and mixed well.

Optionally, standard hybridization buffer can be supplemented with tetramethylammonium chloride (TMACl) or betaine (N,N,N-trimethylglycine; $(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COO}^-$) to improve discrimination between perfectly matched targets and single-base mismatches. Betaine is zwitterionic at neutral pH and alters the composition-dependent stability of nucleic acids without altering their polyelectrolyte behavior. Betaine is preferably used at a concentration between 1 and 10 M and, optimally, at about 5 M. For example, 5 M betaine in 2x SSPE is suitable. Inclusion of betaine at this concentration lowers the average hybridization signal about four fold, but increases the discrimination between matched and mismatched probes.

The scan is performed in the presence of the labeled target. Fig. 21 illustrates an illustrative detection system

for scanning a DNA chip. A series of scans at 30 min intervals using a hybridization temperature of 25°C yields a very clear signal, usually in at least 30 min to two hours, but it may be desirable to hybridize longer, i.e., overnight.

5 Using a laser power of 50 μ W and 50 μ m pixels, one should obtain maximum counts in the range of hundreds to low thousands/pixel for a new slide. When finished, the slide can be stripped using 50% formamide. rinsing well in deionized H₂O, blowing dry, and storing at room temperature.

10 C. PREPARATION OF LABELED RNA/HYBRIDIZATION TO ARRAY

1. TAGGED PRIMERS

The primers used to amplify the target nucleic acid should have promoter sequences if one desires to produce RNA from the amplified nucleic acid. Suitable promoter sequences
15 are shown below and include:

- (1) the T3 promoter sequence:
5'-CGGAATTAACCCTCACTAAAGG
5'-AATTAACCCTCACTAAAGGGAG;
(2) the T7 promoter sequence:
20 5' TAATACGACTCACTATAGGGAG;
and (3) the SP6 promoter sequence:
5' ATTTAGGTGACACTATAGAA.

The desired promoter sequence is added to the 5' end of the PCR primer. It is convenient to add a different
25 promoter to each primer of a PCR primer pair so that either strand may be transcribed from a single PCR product.

Synthesize PCR primers so as to leave the DMT group on. DMT-on purification is unnecessary for PCR but appears to be important for transcription. Add 25 μ l 0.5M NaOH to
30 collection vial prior to collection of oligonucleotide to keep the DMT group on. Deprotect using standard chemistry -- 55°C overnight is convenient.

HPLC purification is accomplished by drying down the oligonucleotides, resuspending in 1 mL 0.1 M TEAA (dilute 2.0
35 M stock in deionized water, filter through 0.2 micron filter) and filter through 0.2 micron filter. Load 0.5 mL on reverse phase HPLC (column can be a Hamilton PRP-1 semi-prep, #79426). The gradient is 0 -> 50% CH₃CN over 25 min (program 0.2 μ mol.prep.0-50, 25 min). Pool the desired fractions, dry

down, resuspend in 200 μ l 80% HAc. 30 min RT. Add 200 μ l EtOH; dry down. Resuspend in 200 μ l H₂O, plus 20 μ l NaAc pH5.5, 600 μ l EtOH. Leave 10 min on ice; centrifuge 12,000 rpm for 10 min in microfuge. Pour off supernatant. Rinse
5 pellet with 1 mL EtOH, dry, resuspend in 200 μ l H₂O. Dry, resuspend in 200 μ l TE. Measure A260, prepare a 10 pmol/ μ l solution in TE (10 mM Tris.Cl pH 8.0, 0.1 mM EDTA). Following HPLC purification of a 42 mer, a yield in the vicinity of 15 nmol from a 0.2 μ mol scale synthesis is typical.

10 2. GENOMIC DNA PREPARATION

Add 500 μ l (10 mM Tris.Cl pH8.0, 10 mM EDTA, 100 mM NaCl, 2% (w/v) SDS, 40 mM DTT, filter sterilized) to the sample. Add 1.25 μ l 20 mg/ml proteinase K (Boehringer) Incubate at 55°C for 2 hours, vortexing once or twice.

15 Perform 2x 0.5 mL 1:1 phenol:CHCl₃ extractions. After each extraction, centrifuge 12,000 rpm 5 min in a microfuge and recover 0.4 mL supernatant. Add 35 μ l NaAc pH5.2 plus 1 mL EtOH. Place sample on ice 45 min; then centrifuge 12,000 rpm 30 min, rinse, air dry 30 min, and resuspend in 100 μ l TE.

20 3. PCR

PCR is performed in a mixture containing, per reaction: 1 μ l genomic DNA; 4 μ l each primer (10 pmol/ μ l stocks); 4 μ l 10 x PCR buffer (100 mM Tris.Cl pH8.5, 500 mM KCl, 15 mM MgCl₂); 4 μ l 2 mM dNTPs (made from 100 mM dNTP
25 stocks); 1 U Taq polymerase (Perkin Elmer, 5 U/ μ l); H₂O to 40 μ l. About 40 cycles (94°C 30 sec, 55°C 30 sec, 72°C 30 sec) are performed, but cycling conditions may need to be varied. These conditions are for 0.2 mL thin wall tubes in Perkin Elmer 9600. For products in the 200 to 1000 bp size range,
30 check 2 μ l of the reaction on a 1.5% 0.5xTBE agarose gel using an appropriate size standard. For larger or smaller volumes (20 - 100 μ l), one can use the same amount of genomic DNA but adjust the other ingredients accordingly.

 4. IN VITRO TRANSCRIPTION

35 Mix: 3 μ l PCR product; 4 μ l 5x buffer; 2 μ l DTT; 2.4 μ l 10 mM rNTPs (100 mM solutions from Pharmacia); 0.48 μ l 10 mM fluorescein-UTP (Fluorescein-12-UTP, 10 mM solution, from Boehringer Mannheim); 0.5 μ l RNA polymerase (Promega T3

or T7 RNA polymerase); and add H₂O to 20 μ l. Incubate at 37°C for 3 h. Check 2 μ l of the reaction on a 1.5% 0.5xTBE agarose gel using a size standard. 5x buffer is 200 mM Tris pH 7.5, 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl, and 100 mM DTT (supplied with enzyme). The PCR product needs no purification and can be added directly to the transcription mixture. A 20 μ l reaction is suggested for an initial test experiment and hybridization; a 100 μ l reaction is considered "preparative" scale (the reaction can be scaled up to obtain more target).

10 The amount of PCR product to add is variable; typically a PCR reaction will yield several picomoles of DNA. If the PCR reaction does not produce that much target, then one should increase the amount of DNA added to the transcription reaction (as well as optimize the PCR). The ratio of fluorescein-UTP

15 to UTP suggested above is 1:5, but ratios from 1:3 to 1:10 - all work well. One can also label with biotin-UTP and detect with streptavidin-FITC to obtain similar results as with fluorescein-UTP detection.

For nondenaturing agarose gel electrophoresis of

20 RNA, note that the RNA band will normally migrate somewhat faster than the DNA template band, although sometimes the two bands will comigrate. The temperature of the gel can effect the migration of the RNA band. The RNA produced from in vitro transcription is quite stable and can be stored for months (at

25 least) at -20°C without any evidence of degradation. It can be stored in unsterilized 6xSSPE 0.1% triton X-100 at -20°C for days (at least) and reused twice (at least) for hybridization, without taking any special precautions in preparation or during use. RNase contamination should of

30 course be avoided. When extracting RNA from cells, it is preferable to work very rapidly and to use strongly denaturing conditions. Avoid using glassware previously contaminated with RNases. Use of new disposable plasticware (not necessarily sterilized) is preferred, as new plastic tubes,

35 tips, etc., are essentially RNase free. Treatment with DEPC or autoclaving is typically not necessary.

5. FRAGMENTATION

Heat transcription mixture at 94 degrees for forty min. The extent of fragmentation is controlled by varying Mg^{2+} concentration (30 mM is typical), temperature, and duration of heating.

5 6. HYBRIDIZATION, SCANNING & STRIPPING

A blank scan of the slide in hybridization buffer only is helpful to check that the slide is ready for use. The buffer is removed from the flow cell and replaced with 1 mL of (hydrolysed) RNA in hybridization buffer and mixed well.

10 Incubate for 15-30 min at 18°C. Remove the hybridization solution, which can be saved for subsequent experiments. Rinse the flow cell 4-5 times with fresh changes of 6 x SSPE 0.1% Triton X-100, equilibrated to 18°C. The rinses can be performed rapidly, but it is important to empty the flow cell

15 before each new rinse and to mix the liquid in the cell thoroughly. A series of scans at 30 min intervals using a hybridization temperature of 25°C yields a very clear signal, usually in at least 30 min to two hours, but it may be desirable to hybridize longer, i.e., overnight. Using a laser

20 power of 50 μW and 50 μm pixels, one should obtain maximum counts in the range of hundreds to low thousands/pixel for a new slide. When finished, the slide can be stripped using warm water.

These conditions are illustrative and assume a probe

25 length of ~15 nucleotides. The stripping conditions suggested are fairly severe, but some signal may remain on the slide if the washing is not stringent. Nevertheless, the counts remaining after the wash should be very low in comparison to the signal in presence of target RNA. In some cases, much

30 gentler stripping conditions are effective. The lower the hybridization temperature and the longer the duration of hybridization, the more difficult it is to strip the slide. Longer targets may be more difficult to strip than shorter targets.

35 7. AMPLIFICATION OF SIGNAL

A variety of methods can be used to enhance detection of labelled targets bound to a probe on the array. In one embodiment, the protein MutS (from E. coli) or

equivalent proteins such as yeast MSH1, MSH2, and MSH3; mouse Rep-3, and Streptococcus Hex-A, is used in conjunction with target hybridization to detect probe-target complex that contain mismatched base pairs. The protein, labeled directly or indirectly, can be added to the chip during or after hybridization of target nucleic acid, and differentially binds to homo- and heteroduplex nucleic acid. A wide variety of dyes and other labels can be used for similar purposes. For instance, the dye YOYO-1 is known to bind preferentially to nucleic acids containing sequences comprising runs of 3 or more G residues.

8. DETECTION OF REPEAT SEQUENCES

In some circumstances, i.e., target nucleic acids with repeated sequences or with high G/C content, very long probes are sometimes required for optimal detection. In one embodiment for detecting specific sequences in a target nucleic acid with a DNA chip, repeat sequences are detected as follows. The chip comprises probes of length sufficient to extend into the repeat region varying distances from each end. The sample, prior to hybridization, is treated with a labelled oligonucleotide that is complementary to a repeat region but shorter than the full length of the repeat. The target nucleic acid is labelled with a second, distinct label. After hybridization, the chip is scanned for probes that have bound both the labelled target and the labelled oligonucleotide probe; the presence of such bound probes shows that at least two repeat sequences are present.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

WHAT IS CLAIMED IS:

1. A method of determining copy number of a gene present in an individual, comprising:
analyzing a plurality of polymorphic sites in a
5 chromosome containing a gene from an individual to determine the number of different polymorphic forms present at each site; and
assigning the copy number of the gene as the highest number of polymorphic forms present at a single site.
- 10 2. The method of claim 1, wherein the plurality of polymorphic sites are in a noncoding segment of the gene.
3. The method of claim 1, wherein the plurality of polymorphic sites are silent polymorphisms.
4. The method of claim 3, wherein the at least one
15 polymorphic site is present in an intronic segment of the gene.
5. The method of claim 1, wherein the plurality of polymorphic sites comprises at least 10 sites.
6. The method of claim 1, wherein the plurality of
20 polymorphic sites comprises at least 50 sites.
7. The method of claim 1, further comprising:
obtaining a tissue sample from the individual containing the gene and amplifying the gene or a fragment thereof.
8. The method of claim 1, wherein the analyzing
25 comprises:
contacting a nucleic acid comprising the gene or a fragment thereof with an array of oligonucleotides, the array comprising a plurality of subarrays, each subarray spanning a polymorphic site and complementarity to at least one
30 polymorphic form of the gene at the site;

detecting hybridization intensities of the nucleic acid to the oligonucleotides in the array, whereby the pattern of hybridization indicates the number of polymorphic forms present at each polymorphic site.

5 9. The method of claim 8, wherein the subarrays each comprise a plurality of probe groups, each probe group complementarity to a different polymorphic form at the site.

10 10. The method of claim 9, wherein a probe group comprises

(a) a first probe set comprising a plurality of probes spanning a polymorphic site of the gene, each probe comprising a segment of at least six nucleotides exactly complementary to a polymorphic form of the gene at the site, the segment including at least one interrogation position complementary to
15 a corresponding nucleotide in the polymorphic form,

(b) a second probe set comprising a corresponding probe for each probe in the first probe set, the corresponding probe in the second probe set being identical to a sequence comprising the corresponding probe from the first probe set or
20 a subsequence of at least six nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets.

25 11. The method of claim 9, wherein a probe group comprises

(a) a first probe set comprising a plurality of probes spanning a polymorphic site, each probe comprising a segment of at least six nucleotides exactly complementary to a
30 subsequence of a polymorphic form at the site, the segment including at least one interrogation position complementary to a corresponding nucleotide in the polymorphic form,

(b) second, third and fourth probe sets, each comprising a corresponding probe for each probe in the first probe set,
35 the probes in the second, third and fourth probe sets being

identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least six nucleotides thereof that includes the at least one interrogation position, except that the at least one
5 interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe sets.

12. The method of claim 1, wherein a single polymorphic form is present at each of the plurality of sites and the copy
10 number of the gene is assigned as 1.

13. The method of claim 1, wherein two polymorphic forms are present at one site and a single polymorphic form is present at each other of the plurality of sites, and the copy number of the gene is assigned as 2.

15 14. The method of claim 1, wherein three polymorphic forms are present at a first polymorphic site, a single polymorphic form is present at a second polymorphic site and two polymorphic forms are present at a third polymorphic site and the copy number of the gene is assigned as 3.

20 15. The method of claim 1, further comprising analyzing a phenotype-determining polymorphic site in the gene to determine which polymorphic form(s) are present at the site.

16. The method of claim 15, further comprising diagnosing a phenotype of the patient based on the polymorphic
25 form(s) present at the phenotype-determining polymorphic site.

17. A method of analyzing a polymorphic site in a gene of an individual, comprising

(a) hybridizing a sample comprising a target nucleic acid comprising one or more alleles of the gene to an array of
30 oligonucleotide probes immobilized on a solid support, the array comprising:

(1) a first probe set comprising a plurality of probes, each probe comprising a segment of at least six nucleotides exactly complementary to a reference form of the gene, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference form of the gene, the reference form of the gene having a silent polymorphic site and a site of potential mutation associated with a phenotypic change;

(2) second, third and fourth probe sets, each comprising a corresponding probe for each probe in the first probe set, the probes in the second, third and fourth probe sets being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least six nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe sets; and

(b) determining which probes, relative to one another, bind to the target nucleic acid, whereby the relative binding of probes having an interrogation position aligned with the silent polymorphism indicates the number of different alleles of the gene in the sample and the relative binding of probes having an interrogation position aligned with the mutation indicates whether the mutation is present in at least one of the alleles.

18. The method of claim 17, wherein the determining comprises:

(1) comparing the relative specific binding of four corresponding probes from the first, second, third and fourth probe sets;

(2) assigning a nucleotide in the target sequence as the complement of the interrogation position of the probe having the greatest specific binding;

(3) repeating (1) and (2) until each nucleotide of interest in the target sequence has been assigned.

19. An array of oligonucleotide probes immobilized on a solid support, the array comprising at least two sets of oligonucleotide probes,

(1) a first probe set comprising a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of a reference sequence, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence,

(2) a second probe set comprising a corresponding probe for each probe in the first probe set, the corresponding probe in the second probe set being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets;

wherein the probes in the first probe set have at least three interrogation positions respectively corresponding to each of three contiguous nucleotides in the reference sequence;

provided that the array does not contain a complete set of probes of a given length;

wherein the reference sequence is from a biotransformation gene.

20. An array of oligonucleotide probes immobilized on a solid support, the array comprising at least four sets of oligonucleotide probes,

(1) a first probe set comprising a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of a reference sequence, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence,

(2) second, third and fourth probe sets, each comprising a corresponding probe for each probe in the first

probe set, the probes in the second, third and fourth probe sets being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe sets;

provided the array lacks a complete set of probes of a given length;

wherein the reference sequence is from a biotransformation gene.

21. The array of claim 19 or 20, wherein the reference sequence is from a gene encoding an enzyme selected from the group consisting of a cytochrome P450, N-acetyl transferase II, glucose 6-phosphate dehydrogenase, pseudocholinesterase, catechol-O-methyl transferase, and dihydropyridine dehydrogenase.

22. The array of claim 21, wherein the reference sequence is from a gene encoding an enzyme selected from the group consisting of a cytochrome P450, N-acetyl transferase II, glucose 6-phosphate dehydrogenase, pseudocholinesterase, catechol-O-methyl transferase, and dihydropyridine dehydrogenase.

23. The array of claim 22, wherein the enzyme is P450 2D6 or P450 2C19.

24. The array of claim 19 or 20, wherein the reference sequence includes a site of a mutation and a site of a silent polymorphism.

25. The array of claim 24, wherein the silent polymorphism is in an intron or flanking region of a gene.

26. The array of claim 19 or 20, wherein the first probe set has at least 3 interrogation positions respectively corresponding to each of 3 contiguous nucleotides in the reference sequence.

27. The oligonucleotide array of claim 19 or 20, wherein the array has between 100 and 100,000 probes.

28. The oligonucleotide array of claim 19 or 20, wherein the probes are linked to the support via a spacer.

29. The oligonucleotide array of claim 19 or 20, wherein the segment in each probe of the first probe set that is exactly complementary to the subsequence of the reference sequence is 9-21 nucleotides.

30. An array of oligonucleotide probes immobilized on a solid support, the array comprising at least one pair of first and second probe groups, each group comprising a first and second sets of oligonucleotide probes as defined by claim 19;

wherein each probe in the first probe set from the first group is exactly complementary to a subsequence of a first reference sequence and each probe in the first probe set from the second group is exactly complementary to a subsequence from a second reference sequence.

31. The array of claim 30, wherein each group further comprises third and fourth probe sets, each comprising a corresponding probe for each probe in the first probe set, the probes in the second, third and fourth probe sets being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the interrogation position, except that the interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe sets.

32. The array of claim 30, wherein the first reference sequence includes the site of a mutation in the biotransformation gene, and the second reference sequence includes a site of a silent polymorphism within the biotransformation gene or flanking the biotransformation gene.

33. The array of claim 32, wherein the reference sequence is from a gene encoding an enzyme selected from the group consisting of a cytochrome P450, N-acetyl transferase II, glucose 6-phosphate dehydrogenase, pseudocholinesterase, catechol-O-methyl transferase, and dihydropyridine dehydrogenase.

34. The array of claim 32 that comprises at least forty pairs of first and second probe groups, wherein the probes in the first probe sets from the first groups of the forty pairs are exactly complementary to subsequences from forty respective first reference sequences.

35. A block of oligonucleotide probes immobilized on a solid support, comprising:

a perfectly matched probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of a reference sequence, the segment having a plurality of interrogation positions respectively corresponding to a plurality of nucleotides in the reference sequence,

for each interrogation position, three mismatched probes, each identical to a sequence comprising the perfectly matched probe or a subsequence of at least three nucleotides thereof including the plurality of interrogation positions, except in the interrogation position, which is occupied by a different nucleotide in each of the three mismatched probes and the perfectly matched probe;

provided the array lacks a complete set of probes of a given length;

wherein the reference sequence is from a biotransformation gene.

36. The array of claim 34, wherein the segment of the perfectly matched probe comprises 3-20 interrogation positions corresponding to 3-20 respective nucleotides in the reference sequence.

37. An array of probes immobilized to a solid support comprising at least two blocks of probes, each block as defined by claim 34, a first block comprising a perfectly matched probe comprising a segment exactly complementary to a subsequence of a first reference sequence and a second block comprising a perfectly matched probe comprising a segment exactly complementary to a subsequence of a second reference sequence.

38. The array of claim 37, wherein the first reference sequence is from a wildtype 2D6 gene and the second reference sequence is from a mutant 2D6 gene.

39. The array of claim 37, comprising at least 10-100 blocks of probes, each comprising a perfectly matched probe comprising a segment exactly complementary to a subsequence of at least 10-100 respective reference sequences.

40. An array of oligonucleotide probes immobilized on a solid support, the array comprising at least four probes:

a first probe comprising first and second segments, each of at least three nucleotides and exactly complementary to first and second subsequences of a reference sequence, the segments including at least one interrogation position corresponding to a nucleotide in the reference sequence, wherein either (1) the first and second subsequences are noncontiguous, or (2) the first and second subsequences are contiguous and the first and second segments are inverted relative to the complement of the first and second subsequences in the reference sequence;

second, third and fourth probes, identical to a sequence comprising the first probe or a subsequence thereof comprising at least three nucleotides from each of the first and second

segments, except in the at least one interrogation position, which differs in each of the probes;

provided the array lacks a complete set of probes of a given length;

wherein the reference sequence is from a biotransformation gene.

41. A method of comparing a target nucleic acid with a reference sequence comprising a predetermined sequence of nucleotides, the method comprising:

(a) hybridizing a sample comprising the target nucleic acid to an array of oligonucleotide probes immobilized on a solid support, the array comprising:

(1) a first probe set comprising a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of the reference sequence, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence, wherein the reference sequence is from a biotransformation gene;

(2) a second probe set comprising a corresponding probe for each probe in the first probe set, the corresponding probe in the second probe set being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets;

wherein, the probes in the first probe set have at least three interrogation positions respectively corresponding to each of at least three nucleotides in the reference sequence, and

(b) determining which probes, relative to one another, in the first and second probe sets specifically bind to the target nucleic acid, the relative specific binding of corresponding probes in the first and second probe sets indicating whether a nucleotide in the target sequence is the

same or different from the corresponding nucleotide in the reference sequence.

42. The method of claim 41, wherein the determining step comprises:

- (1) comparing the relative specific binding of two corresponding probes from the first and second probe sets;
- (2) assigning a nucleotide in the target sequence as the complement of the interrogation position of the probe having the greater specific binding; and
- (3) repeating (1) and (2) until each nucleotide of interest in the target sequence has been assigned.

43. The method of claim 41, wherein the array further comprises third and fourth probe sets, each comprising a corresponding probe for each probe in the first probe set, the probes in the second, third and fourth probe sets being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe sets; and the determining step comprises determining which probes, relative to one another, in the first, second, third and fourth probe sets specifically bind to the target nucleic acid, the relative specific binding of corresponding probes in the first, second, third and fourth probe sets indicating whether a nucleotide in the target sequence is the same or different from the corresponding nucleotide in the reference sequence.

44. The method of claim 43, wherein:

the reference sequence includes a site of a mutation in the biotransformation gene and a silent polymorphism in or flanking the biotransformation gene;

the target nucleic acid comprises one or more different alleles of the biotransformation gene; and

the relative specific binding of probes having an interrogation position aligned with the silent polymorphism indicates the number of different alleles and the relative specific binding of probes having an interrogation position aligned with the mutation indicates whether the mutation is present in at least one of the alleles.

45. The method of claim 43, wherein the determining comprises:

(1) comparing the relative specific binding of four corresponding probes from the first, second, third and fourth probe sets;

(2) assigning a nucleotide in the target sequence as the complement of the interrogation position of the probe having the greatest specific binding;

(3) repeating (1) and (2) until each nucleotide of interest in the target sequence has been assigned.

46. A method of comparing a target nucleic acid with a reference sequence comprising a predetermined sequence of nucleotides, the method comprising:

(a) hybridizing the target nucleic acid to an array of oligonucleotide probes immobilized on a solid support, the array comprising:

a perfectly matched probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of a reference sequence, the segment having a plurality of interrogation positions respectively corresponding to a plurality of nucleotides in the reference sequence, wherein the reference sequence is from a biotransformation gene;

for each interrogation position, three mismatched probes, each identical to a sequence comprising the perfectly matched probe or a subsequence of at least three nucleotides thereof including the plurality of interrogation positions, except in the interrogation position, which is occupied by a different nucleotide in each of the three mismatched probes and the perfectly matched probe;

(b) for each interrogation position,

- (1) comparing the relative specific binding of the three mismatched probes and the perfectly matched probe;
- (2) assigning a nucleotide in the target sequence as the complement of the interrogation position of the probe having the greatest specific binding.

47. The method of claim 46, wherein the target sequence has an undetermined substitution relative to the reference sequence, and the method assigns a nucleotide to the substitution.

48. A method of screening a patient for capacity to metabolize a drug, the method comprising:

(a) hybridizing a tissue sample from the patient containing a target nucleic acid to an array of oligonucleotide probes immobilized on a solid support, the array comprising:

(1) a first probe set comprising a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of the reference sequence from a biotransformation gene which metabolizes the drug, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence,

(2) a second probe set comprising a corresponding probe for each probe in the first probe set, the corresponding probe in the second probe set being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets;

wherein, the probes in the first probe set have at least three interrogation positions respectively corresponding to each of at least three nucleotides in the reference sequence, and

(b) determining which probes, relative to one another, in the first and second probe sets specifically to the target nucleic acid, the relative specific binding of corresponding probes in the first and second probe sets indicating whether the target sequence contains a mutation relative to the reference sequence, which, if present, impairs the capacity of the patient to metabolize the drug.

49. A method of conducting a clinical trial on a drug, the method comprising:

(a) obtaining a tissue sample containing a target nucleic acid from each of a pool of patients;

(b) for each tissue sample, hybridizing the target nucleic acid to an array of oligonucleotide probes immobilized on a solid support, the array comprising:

(1) a first probe set comprising a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of the reference sequence from a biotransformation gene, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence,

(2) a second probe set comprising a corresponding probe for each probe in the first probe set, the corresponding probe in the second probe set being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets;

wherein, the probes in the first probe set have at least three interrogation positions respectively corresponding to each of at least three nucleotides in the reference sequence;

(c) determining which probes, relative to one another, in the first and second probe sets specifically to the target nucleic acid, the relative specific binding of corresponding probes in the first and second probe sets

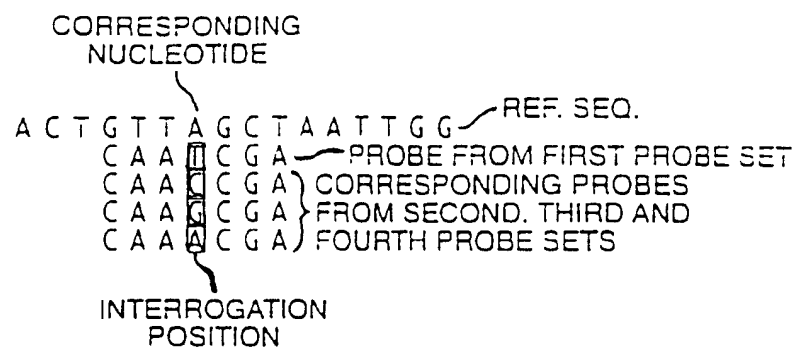
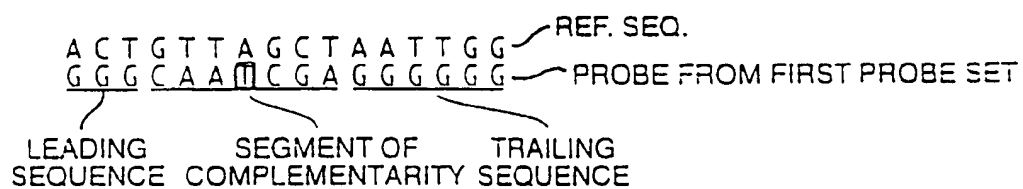
indicating whether the target sequence contains a mutation relative to the reference sequence

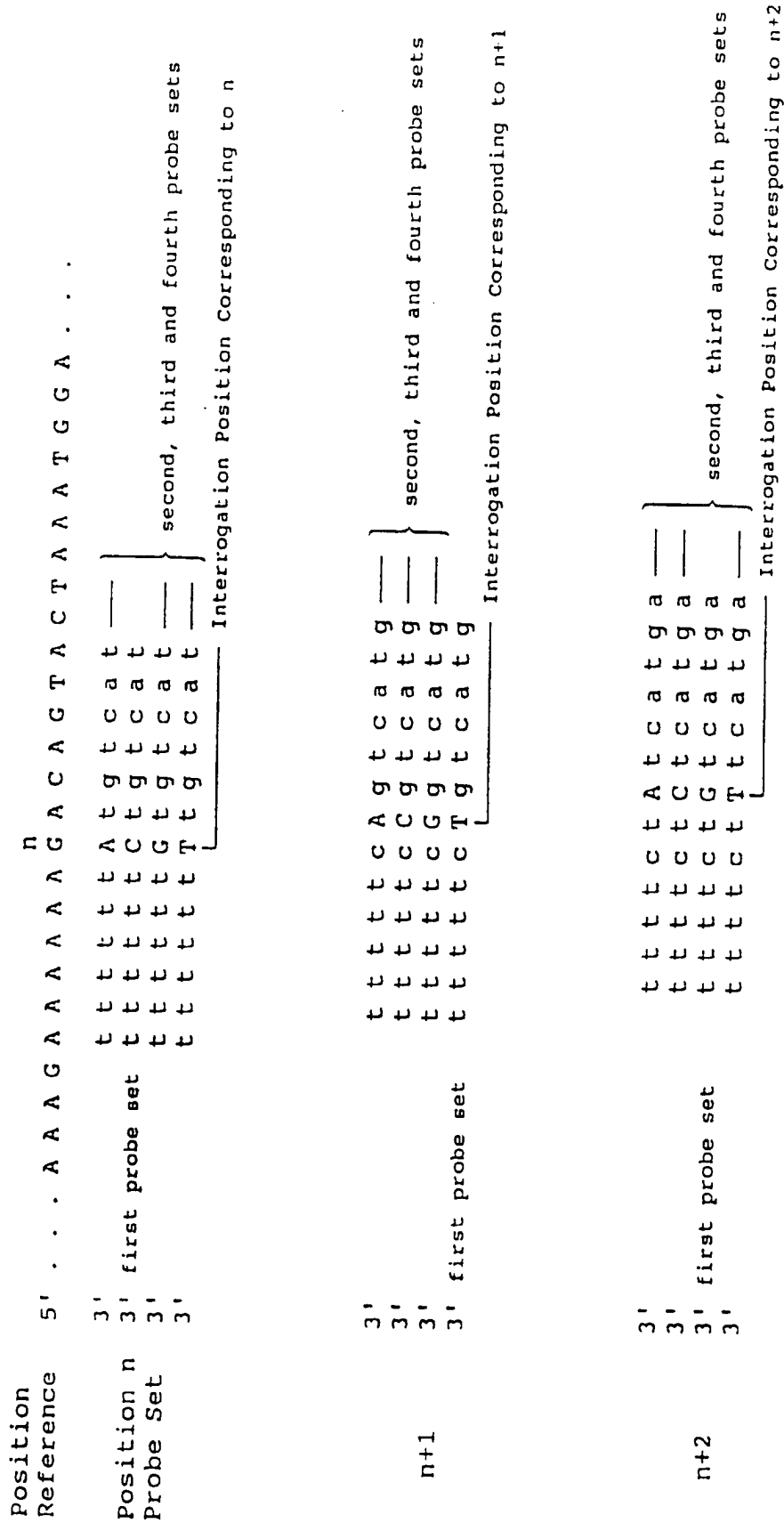
selecting a subpool of patients having a target sequence free of the mutation; and

(d) administering the drug to the subpool of patients to determine efficacy.

50. The method of claim 49, further comprising combining the drug with a pharmaceutical carrier to form a pharmaceutical composition.

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**FIG. 1****FIG. 2**



3B

FIG. 4A

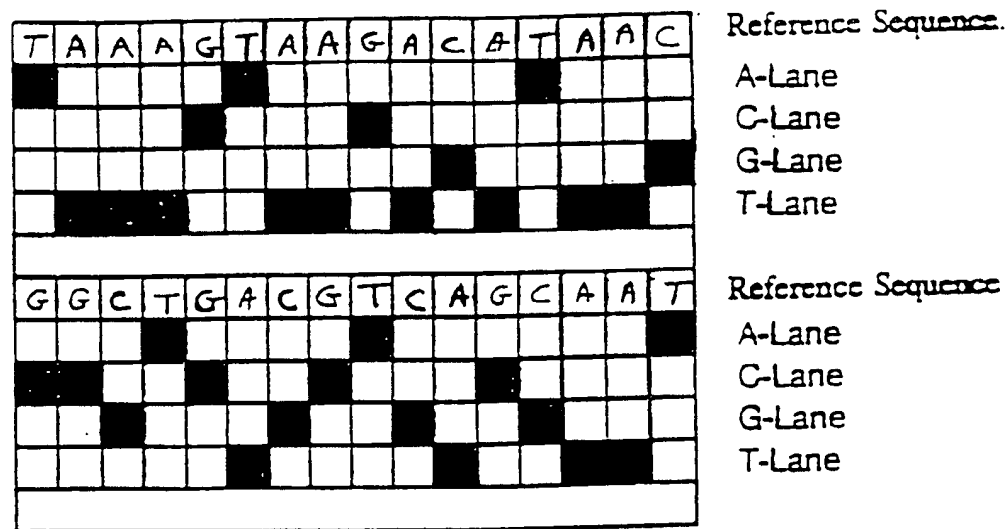
FIG. 4A

G G G X C C C T T A A

C	C	C	A	G	G	G
C	C	C	C	G	G	G
C	C	C	G	G	G	G
C	C	C	T	G	G	G

A	G	G	A	A	A	T
C	G	G	G	A	A	T
G	G	G	G	A	A	T
T	G	G	G	A	A	T

FIG. 4C



Tiled Array with Probes for the Detection
of Point Mutations

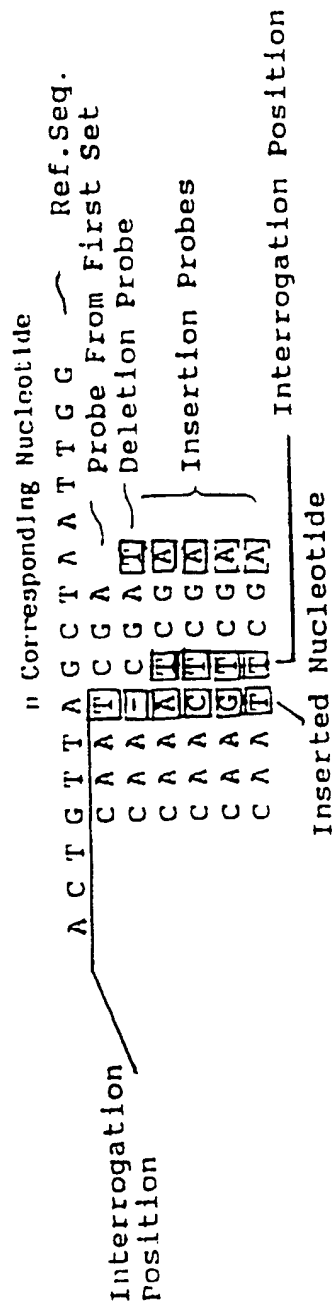


FIG. 6

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n_1 n_2 n_3 — CORRESPONDING NUCLEOTIDES
 A C T G T T A G C T A A T T G G — REF. SEQ.
 C A A C C A — PROBE FROM FIRST SET
 l_1 l_2 l_3 — INTERROGATION POSITIONS

C A T C G A } CORRESPONDING PROBES
 C G A T C G A } FROM SECOND, THIRD AND
 C A T C G A } FOURTH PROBE SETS
 l_1

C A A C G A } CORRESPONDING PROBES
 C A A C G A } FROM FIFTH, SIXTH AND
 C A A C G A } SEVENTH PROBE SETS
 l_2

C A A T C A } CORRESPONDING PROBES
 C A A T C A } FROM EIGHTH, NINTH AND
 C A A T C A } TENTH PROBE SETS
 l_3

FIG. 7

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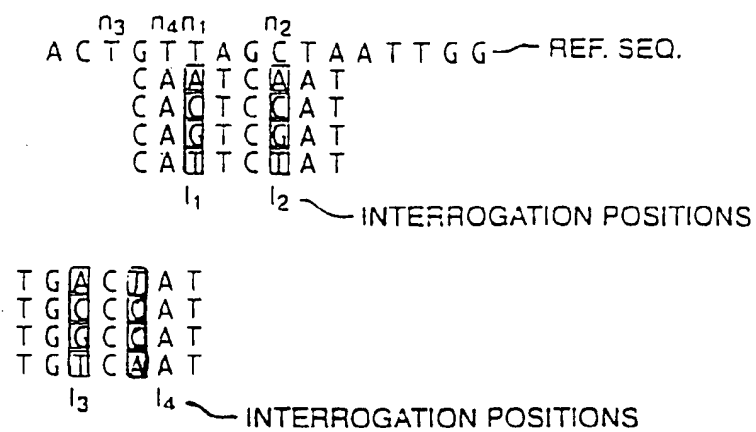


FIG. 8

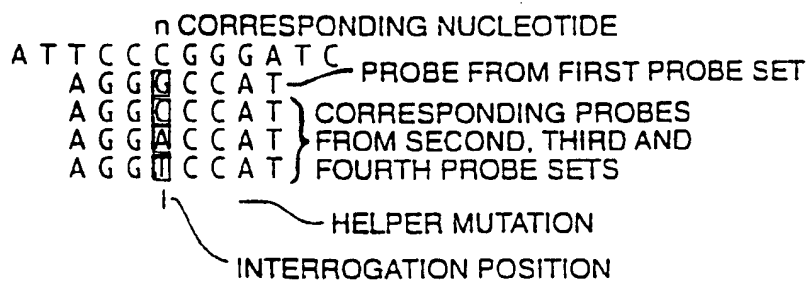
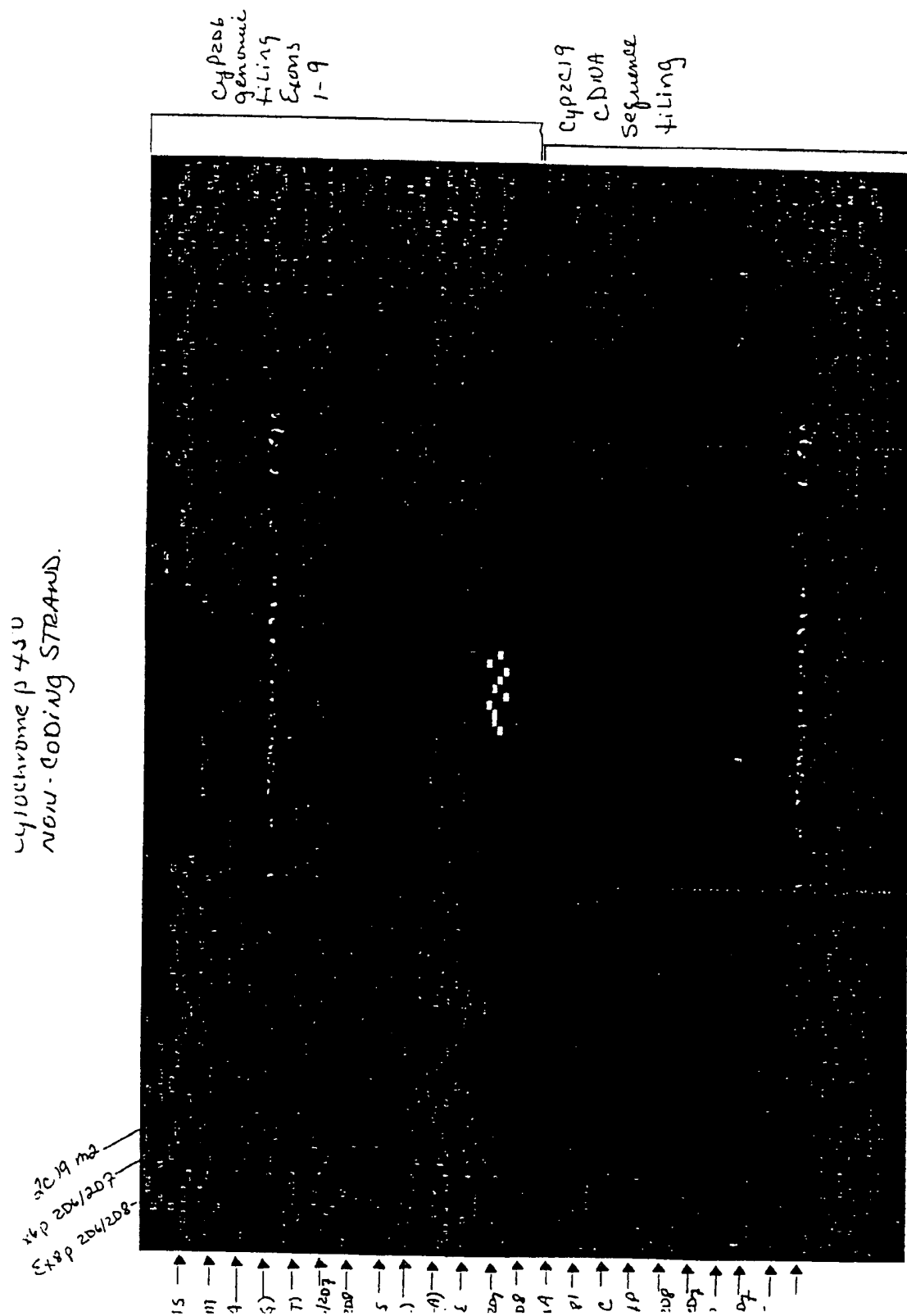


FIG. 9

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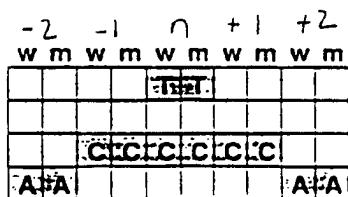


CYP2D6/CYP2D7 POLYMORPHISM DETECTION BLOCK

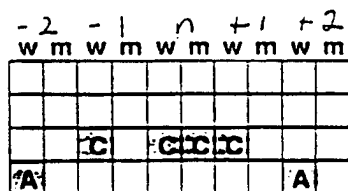
[illegible][illegible][illegible]

FIG 11

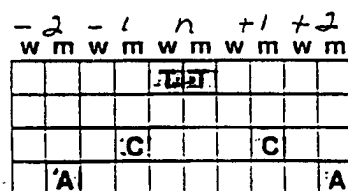
CYP2D6 P34S MUTATION DETECTION BLOCK



P34S Heterozygote Hybridization

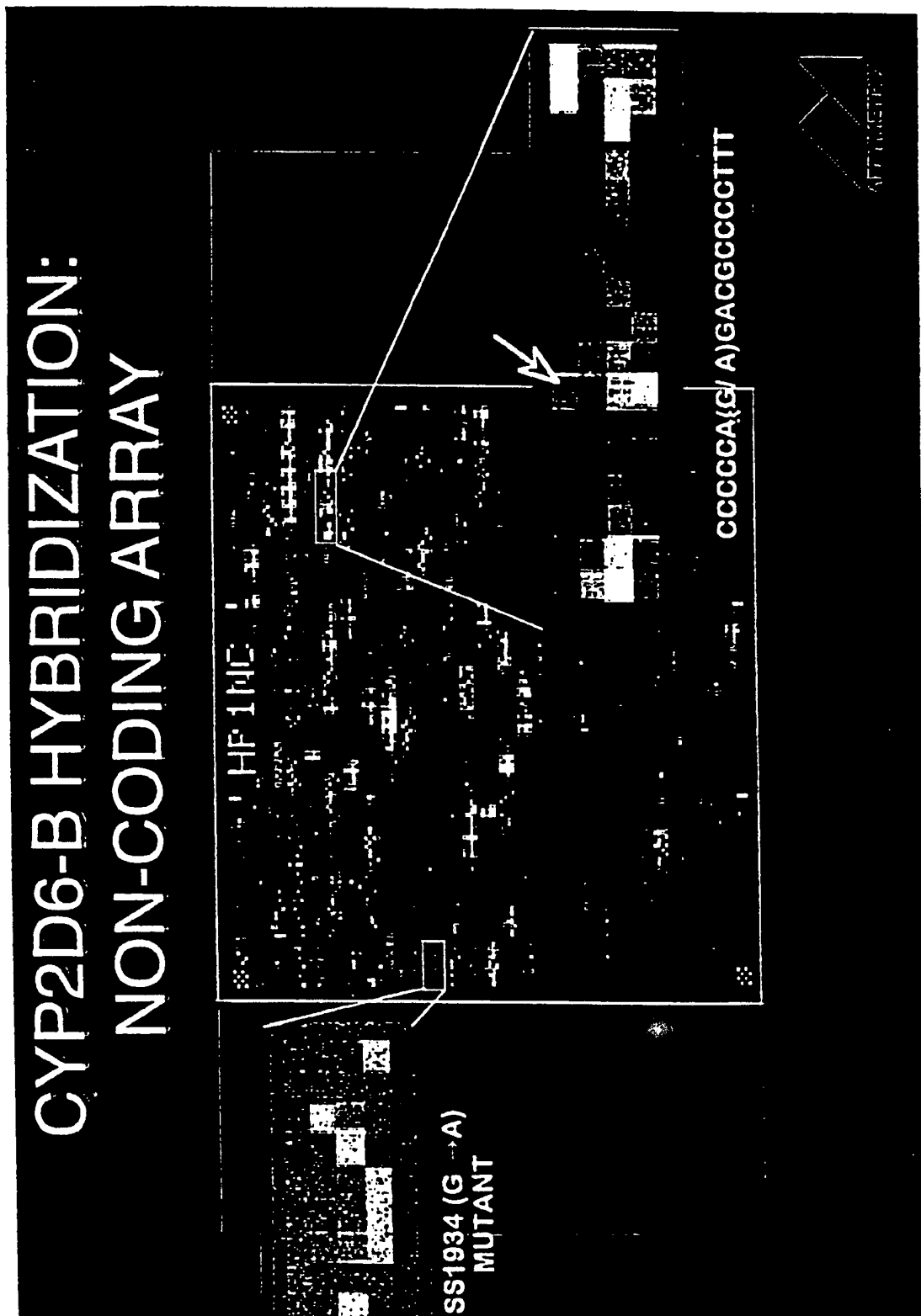


P343S Wild Type Hybridization



P34S Mutant Hybridization

FIG. 12

**FIG. 13**

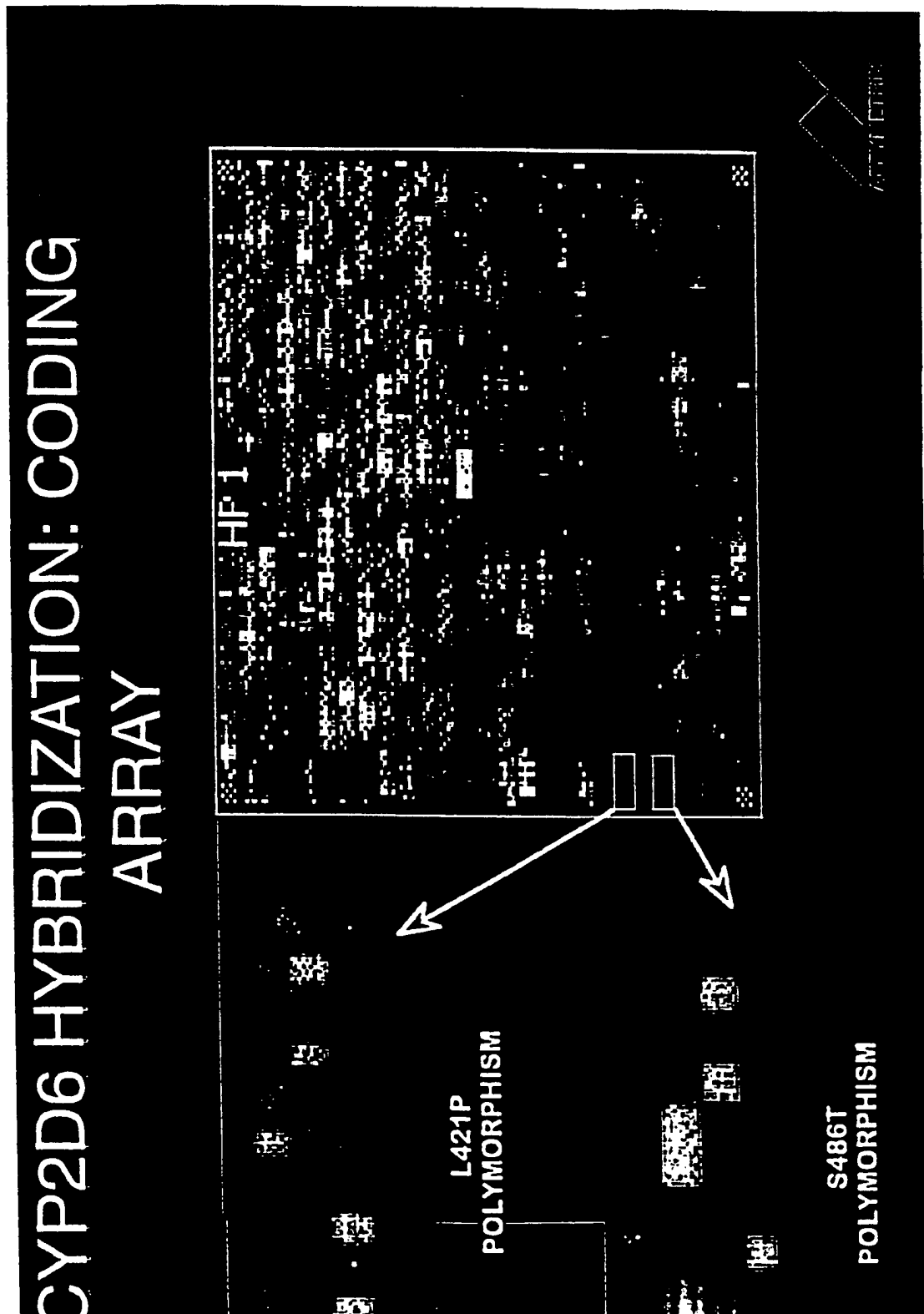


FIG. 14

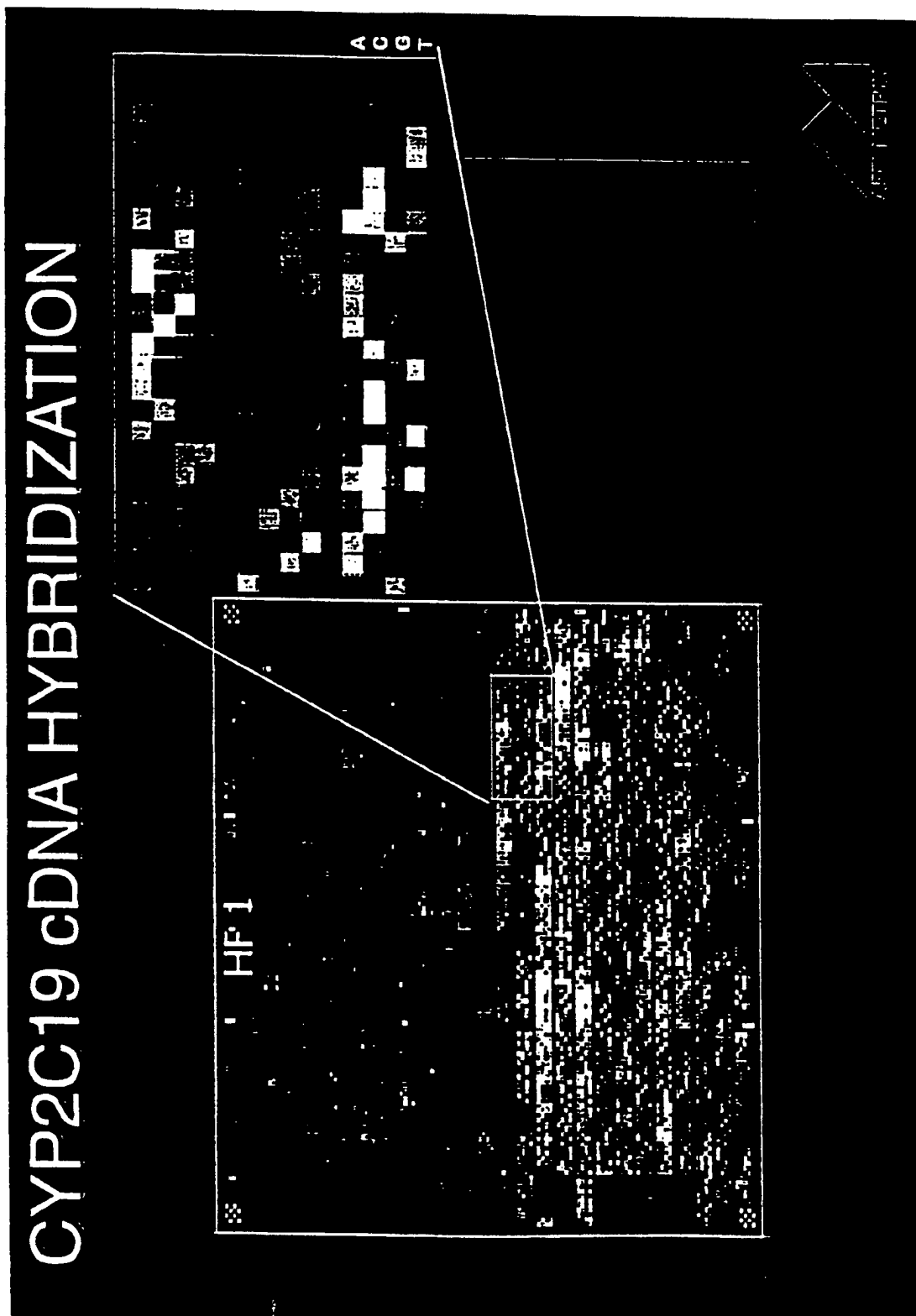


FIG. 15